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chemico-biological interactions

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A. Machino, H. Inano and B. Tamaoki. Studies on enzyme reactions related to steroid biosynthesis — 1. Presence of the cytochrome P-450 in testicular tissue and its role in the biogenesis of androgens.

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BINDING OF ^{14}C-LABELED 4-NITROQUINOLINE 1-OXIDE TO DNA *IN VIVO**

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(Received December 4th, 1969)

SUMMARY

The mode of binding of a carcinogen, 4-nitroquinoline 1-oxide (4NQO) to DNA was investigated by incubating Ehrlich ascites tumor cells growing in the peritoneal cavity of mice with ^{14}C-labeled 4NQO. DNA extracted from incubated cells had radioactivity associated with DNA after neutral and alkaline cesium chloride equilibrium density-gradient centrifugation. The radioactivity was not released from DNA by heat or alkaline denaturation. Digestion of the ^{14}C-associated DNA by deoxyribonuclease and snake venom phosphodiesterase gave a nucleotide compound which was eluted before ordinary mononucleotides on gel filtration. Phosphomonoesterase treatment of this material yielded a compound which was eluted after mononucleotides on gel filtration and which was not adsorbed on DEAE-cellulose column chromatography. Upon depurination of the ^{14}C-associated DNA under mild conditions, almost all the radioactivity was released with purine bases. The data suggest a covalent binding of 4NQO or its derivatives with purine bases of DNA.

INTRODUCTION

4NQO or its derivatives are known mutagens in microorganism[1–3] and also potent carcinogens[4]. In studies of the carcinogenic mechanism of 4NQO, the binding of 4NQO or its derivatives to DNA *in vitro*[5–9] and *in vivo*[10,11] was reported. However, the nature of its binding to DNA has not yet been fully explored.

The present study was undertaken to confirm the binding of the carcinogen

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** On leave of absence from Cancer Institute, Japanese Foundation for Cancer Research, Toshima-ku, Tokyo.

Abbreviations: 4HAQO, 4-hydroxyaminoquinoline 1-oxide; 4NQO, 4-nitroquinoline 1-oxide; SSC, 0.14 *M* NaCl–0.014 *M* sodium citrate.

to DNA *in vivo* by using [^{14}C]4NQO and Ehrlich ascites tumor cells as a mode, system. The chemical properties of the carcinogen–DNA complex were investigated.

MATERIALS AND METHODS

Chemicals and enzymes

[5,6,7,8,9,10-^{14}C]4NQO (specific radioactivity 7.76 mCi/mmole) and carrier-free [^{32}P]orthophosphate were purchased from Daiichi Chemical Co., Tokyo. [^{14}C]4NQO gave a single peak on thin-layer chromatography in several solvent systems. [^{32}P]Orthophosphate was boiled in 1 *N* HCl for 30 min to hydrolyze contaminating polyphosphate and then neutralized with NaOH.

Bovine pancreatic deoxyribonuclease, snake venom phosphodiesterase, *E. coli* alkaline phosphatase and bovine pancreatic ribonuclease were obtained from Worthington Biochemicals, Freehold, New Jersey. Pronase was kindly donated by Dr. M. NOMOTO, Seikagaku Kogyo Co., Tokyo.

Preparation of DNA

Approximately 10^6 Ehrlich ascites tumor cells were injected intraperitoneally into adult male mice of the ddN strain. 7 days after transplantation, 20 μCi [^{14}C]-4NQO dissolved in a small amount of dimethyl sulfoxide, followed by appropriate dilution with physiologic saline, was injected intraperitoneally into the mice. The mice were killed by decapitation 1 h later, the ascitic fluid was drained and tumor cells were harvested by washing with cold saline to remove contaminating red blood cells. The cells were disrupted with 4 volumes of SSC with a VirTis 45 homogenizer. The homogenate was centrifuged at 4300 rev./min for 10 min and DNA was extracted from the sediment according to the method of KAY *et al.*[12]. For studying the time course of the binding, mice bearing ascites tumor were injected intraperitoneally with 5 μCi [^{14}C]4NQO and DNA was isolated from the tumor cells after various periods of time by the same method.

^{32}P-Labeled DNA was prepared in the same way from the tumor cells of the mice which were injected intraperitoneally with 0.1 mCi carrier-free [^{32}P]orthophosphate.

DNAase, RNAase and pronase digestion of DNA

DNA (1 mg) was incubated with 10 μg DNAase in 1 ml of 50 m*M* Tris–HCl buffer (pH 7.4) with 5 m*M* $MgCl_2$. In the case of pancreatic ribonuclease, 20 μg of the enzyme was used in 1 ml of 50 m*M* Tris–HCl buffer (pH 8.0) with 5 m*M* $MgCl_2$. DNA (1 mg) was treated with 50 μg pronase in 1 ml of 50 m*M* Tris–HCl buffer (pH 7.4).

These mixtures were incubated at 37° for 30 min. Radioactivity insoluble in 5 % trichloroacetic acid was assayed by the filter-paper disk method[13].

Heat and alkaline denaturation of DNA

DNA was heated in boiling water for 10 min in 0.1 × SSC at a concentration

of 200 μg/ml. The solution was immediately chilled in an ice bath and acid-insoluble radioactivity was assayed by the filter disk method.

DNA was dissolved in 0.3 *N* NaOH and incubated at 37° for 18 h. After neutralizing the solution, acid-insoluble radioactivity was assayed by the filter disk method.

CsCl equilibrium density-gradient centrifugation

DNA was analyzed with cesium chloride equilibrium density-gradient centrifugation under neutral and alkaline (0.1 *N* NaOH) conditions, using a Hitachi RPS-40 swinging-bucket type rotor at 37 500 rev./min for 64 h at 25°. In both cases, the volume was adjusted to 5.0 ml and the solution contained 4.85 g of CsCl. 10-drop fractions were collected by puncturing the bottom of the tube with a 22-gauge needle. The absorbance at 260 nm and the radioactivity were assayed.

Enzymatic digestion of DNA and separation of the digest

The DNA at a concentration of 2 mg/ml was digested for 24 h with 100 μg/ml pancreatic deoxyribonuclease plus 100 μg/ml of snake venom phosphodiesterase in 50 m*M* Tris–HCl buffer (pH 7.4) containing 5 m*M* $MgCl_2$. 400 μg of the hydrolysate was applied to a column (1.0 × 47 cm) of Sephadex G-15 (Pharmacia, Uppsala). Elution was performed by 0.01 *M* triethylamine carbonate buffer (pH 7.7). Fractions of 2 ml were collected at a flow rate of 8 ml/h.

Another 2.9 mg of the DNA digest diluted to 8 ml with 0.01 *M* ammonium bicarbonate buffer (pH 8.6) was applied to a column (1.0 × 24 cm) of DEAE-cellulose (Brown, Berlin, New Hampshire), equilibrated with the same buffer. After washing with 30 ml of the buffer, the column was developed with a linear gradient of 0.01–2.0 *M* ammonium bicarbonate buffer (pH 8.6). Fractions of 5 ml were collected at a flow rate of 100 ml/h.

The DNA digest and the radioactive fractions eluted from the Sephadex G-15 column were treated with 1 or 20 μg/ml *E. coli* alkaline phosphatase and the resulting compounds were chromatographed on Sephadex G-15 and DEAE-cellulose columns as described above.

Conditions of depurination

Depurination of the DNA was carried out according to the method of TAMM *et al.*[14]. To a solution of 3 mg DNA in 1.5 ml of water, 0.2 *N* HCl was added gradually to reach pH 1.6 yielding a heavy precipitate. The mixture was made up to 2.0 ml with dilute HCl (pH 1.6), transferred to a cellophane bag and dialyzed against 15 ml of dilute HCl (pH 1.6) for 26 h at 37°. Aliquots were taken at various intervals from the dialysate to determine purine bases and radioactivity. The bag contents were taken to dryness *in vacuo* with a rotary evaporator under 37°. The residue was dissolved in 1.0 ml of 0.2 *M* ammonium formate buffer (pH 4.0) and applied to a column (1.0 × 57 cm) of Sephadex G-50, fine (Pharmacia). Elution was performed with the same buffer and 2-ml fractions were collected at a flow rate of 52 ml/h.

RESULTS

Quinoline binding to DNA of ascites cells

Radioactivity of 1.3 mμCi, corresponding to 0.17 mμmole of quinoline derivative, was bound to 1 mg of DNA extracted from ascites tumor cells 1 h after injection of 20 μCi of [^{14}C]4NQO. This value corresponded to one molecule of quinoline derivative per 10 000 nucleotide pairs of DNA.

The extent of binding of isotope to DNA at various times after injection of [^{14}C]4NQO is shown in Table I. After reaching a maximum 4 h after the administration of [^{14}C]4NQO the label on the DNA decreased gradually afterwards. At 32 h, the specific radioactivity was about one-third of the value at 4 h.

TABLE I

TIME COURSE OF BINDING OF [^{14}C]4NQO TO DNA

Time after 4NQO injection (h)	*DNA specific activity (counts/min/mg DNA)*
2	605
4	755
8	590
16	380
32	280

DNA was isolated from Ehrlich ascites cells in mice at various times after intraperitoneal injection of 5μCi of [^{14}C]NQO.

Enzymatic digestion of ^{14}C-associated DNA

The radioactive material was not converted to an acid-soluble form by incubation with pancreatic ribonuclease or pronase, whereas incubation with pancreatic deoxyribonuclease resulted in the almost complete release of the radioactivity into an acid-soluble form (Table II). This result suggested that the ^{14}C-labeled quinoline compound was clearly associated with DNA but not with any RNA or proteins possibly present as contaminants in the DNA preparation.

Heat and alkaline denaturation of ^{14}C-associated DNA

Table III shows that 20 % of the acid-insoluble radioactivity was converted to an acid-soluble form by heat denaturation. Control experiments using the same amount of [^{32}P]DNA isolated from Ehrlich ascites cells proved that 12 % radioactivity was converted to acid-soluble by heat denaturation. After alkaline denaturation of the ^{14}C-associated DNA and control [^{32}P]DNA, about 90 % radioactivity remained acid-insoluble. These results indicated the binding of the quinoline compound to DNA was not affected by transition of the DNA molecule from a double-stranded to a single-stranded form.

CsCl equilibrium density-gradient centrifugation

Binding of the radioactivity was examined on native and alkaline denatured DNAs by CsCl equilibrium density-gradient centrifugation (Fig. 1). The ultraviolet

TABLE II

ENZYMATIC DIGESTION OF ^{14}C-LABELED QUINOLINE ASSOCIATED DNA

Enzymes	*Radioactivity remaining in acid-insoluble form* (%)
No treatment	100
Pancreatic deoxyribonuclease 10 μg/ml[a]	12
Pancreatic ribonuclease 20 μg/ml[b]	90
Pronase 50 μg/ml[c]	99

DNA at a concentration of 1 mg/ml was incubated with the indicated amount of each enzyme at 37° for 30 min. The incubation medium for the respective enzyme was as follows:

[a] Tris–HCl (pH 7.4) 50 m*M*; $MgCl_2$ 5 m*M*.

[b] Tris–HCl (pH 8.0) 50 m*M*; $MgCl_2$ 5 m*M*.

[c] Tris–HCl (pH 7.4) 50 m*M*.

TABLE III

HEAT AND ALKALINE DENATURATION OF ^{14}C-LABELED QUINOLINE ASSOCIATED DNA

	Acid-insoluble radioactivity	
	Counts/min/mg DNA	%
^{14}C-Associated DNA		
Native	2197	100
Heat denatured[a]	1760	80
Alkaline denatured[b]	2020	92
[^{32}P]DNA		
Native	53470	100
Heat denatured[a]	46900	88
Alkaline denatured[b]	45600	85

[a] DNA (200 μg/ml) in 0.1 × SSC was heated in boiling water for 10 min and cooled immediately in ice.

[b] DNA (1.0 mg/ml) in 0.3 *N* NaOH was incubated at 37° for 18 h. An aliquot of each 0.1 ml was placed on filter-paper disks and the acid-insoluble radioactivity was assayed as described in MATERIALS AND METHODS.

light-absorbing DNA peaks manifestly carried the radioactivity. This and aforementioned results support the view that the quinoline compound was covalently bound to DNA rather than by physical interactions such as implied in an intercalation model.

Exclusion column chromatography

Digestion of ^{14}C-associated DNA with pancreatic deoxyribonuclease plus snake venom phosphodiesterase and subsequent fractionation on a Sephadex G-15 column with 0.01 *M* triethylammonium carbonate buffer (pH 7.7) gave the result shown in Fig. 2. Peak B was found to be a mixture of 4 deoxyribonucleotides by paper chromatography. One-third of the total radioactivity of the enzymatic digest was recovered in peak A.

Treatment of the fraction in peak A with 1 μg/ml of *E. coli* alkaline phosphatase followed by fractionation on the same column gave the result shown in Fig. 3a.

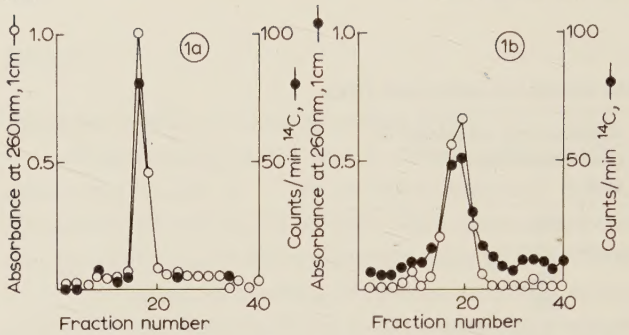


Fig. 1. CsCl equilibrium density-gradient centrifugation of the ^{14}C-labeled quinoline associated DNA from Ehrlich ascites cells. Tube containing DNA (300 μg) was centrifuged at 37500 rev./min for 64 h at 25°. Fig. 1a represents the pattern under neutral condition. Fig. 1b represents the pattern in 0.1 *N* NaOH solution.

Although almost all the monodeoxyribonucleotides of peak A were converted to nucleosides as shown by the shift of the peak of ultraviolet light-absorbing materials, the ^{14}C-radioactivity remained at the same position. The same amount of the peak A fraction was treated with a much higher concentration of the phosphatase (20 μg/ml) for the same period, and the digest was fractionated on a Sephadex G-15 column in the same manner (Fig. 3b). Both peaks of radioactivity and ultraviolet absorption shifted from the original elution position of peak A, and 71% radioactivity of the peak A was eluted between the peaks of nucleotides and nucleosides.

The result suggests that peak A contained a ^{14}C-labeled quinoline associated substance having a free phosphate residue, although it was less susceptible to the phosphatase than the 5′-phosphate of mononucleotides. The molecular size of the substance seemed to be larger than that of mononucleotide.

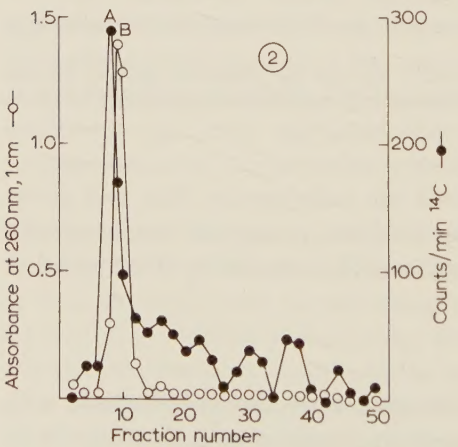


Fig. 2. Elution profile of the enzymatic digest of ^{14}C-labeled quinoline bound DNA on Sephadex G-15. DNA was isolated from the mice bearing Ehrlich ascites cells, which were treated with [^{14}C]4NQO. ^{14}C-Labeled quinoline bound DNA (2 mg/ml, 4000 counts/min) was digested with pancreatic deoxyribonuclease (100 μg/ml) and snake venom phosphodiesterase (100 μg/ml) for 24 h at 37°. The perchloric acid-soluble fraction was neutralized with KOH. After removal of the potassium perchlorate, 1 ml of the hydrolysate which contained 8 absorbance units at 260 nm, was applied on a column (1.0 × 47 cm) of Sephadex G-15. Fractionation was carried out as described in MATERIALS AND METHODS. Blue dextran was eluted at the 6th fraction under the same conditions.

DEAE-cellulose column chromatography

The enzymatic hydrolysate of the ^{14}C-associated DNA with pancreatic DNAase and venom phosphodiesterase was further treated with a high concentration of *E. coli* alkaline phosphatase (20 μg/ml), as described above. The ionic properties of the radioactive substance before and after digestion were investigated by DEAE-cellulose column chromatography. 46% of the radioactivity of the DNA digest with DNAase plus snake venom phosphodiesterase was retained on the column and eluted with 0.2–0.5 *M* ammonium bicarbonate buffer (pH 8.6), although 25% of the radioactivity was not adsorbed on the column (Fig. 4a). In contrast, the radio-

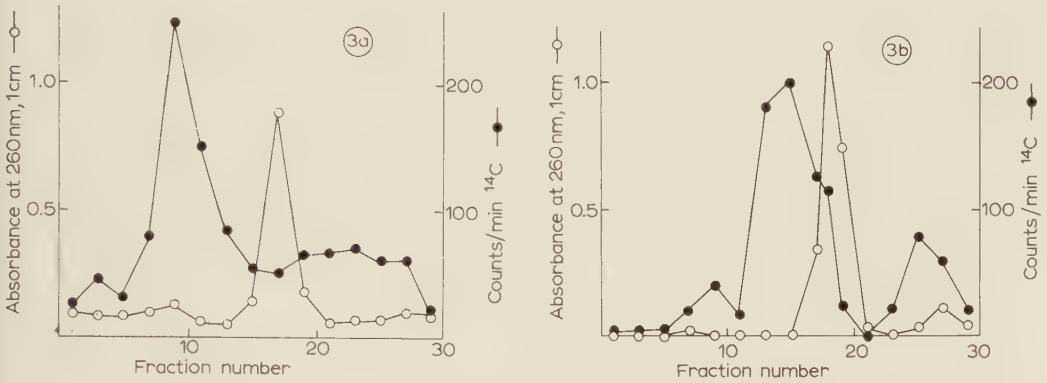


Fig. 3. Elution profile of the phosphomonoesterase digest of the peak A. Tube No. 8 of the chromatogram shown in Fig. 2 on Sephadex G-15 was subjected for digestion by phosphomonoesterase. A portion of the material (0.4 mg/ml) was incubated with *E. coli* alkaline phosphatase, 1 μg/ml (Fig. 3a), or 20 μg/ml (Fig. 3b), and subsequently separated on Sephadex G-15 as described for Fig. 2.

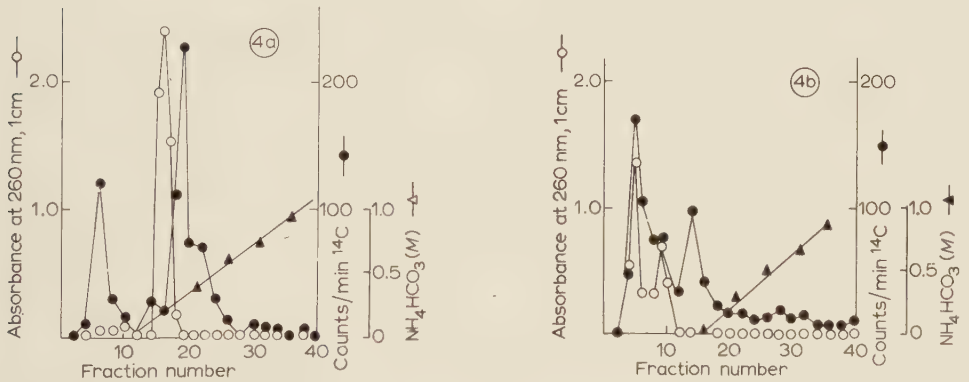
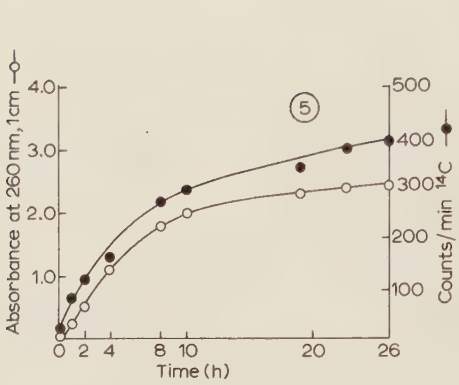


Fig. 4. DEAE-cellulose chromatograms. Fig. 4a represents the elution profile of the same DNA digest shown in Fig. 2. The DNA digest (0.9 mg/ml) was further treated with *E. coli* alkaline phosphatase (20 μg/ml) at 37° for 3 h, and applied to a column of DEAE-cellulose. Fig. 4b represents the elution profile of the phosphatase-treated materials. In both cases, the enzymatic hydrolysates were dissolved in 8 ml of 0.01 *M* ammonium bicarbonate buffer (pH 8.6), and applied to a column (1.0 × 24 cm) of DEAE-cellulose. Elution was carried out as described in MATERIALS AND METHODS.



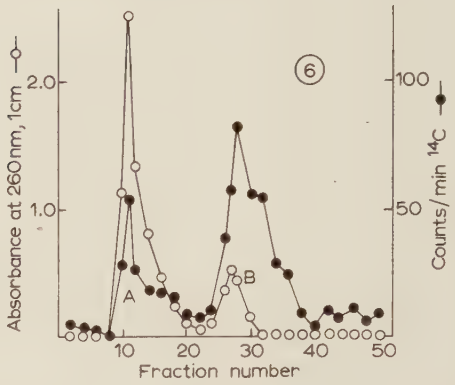


Fig. 5. Kinetics of depurination of ^{14}C-labeled quinoline bound DNA isolated from Ehrlich ascites cells treated with [^{14}C]4NQO. DNA, 3 mg in 2 ml of HCl of pH 1.6 was dialyzed at 37° against 15 ml of the same solution for 26 h. The reaction was performed in a Monod tube with constant shaking. At various intervals, aliquots of 0.75 ml were taken from the dialysate, and the absorbance at 260 nm and the radioactivity were measured.

Fig. 6. Elution profile of the material remaining inside the cellophane bag after depurination on Sephadex G-15. The reaction was performed as described in Fig. 5. After evaporation of the contents of the dialysis bag, the residue was dissolved in 1.0 ml of 0.2 *M* ammonium formate buffer (pH 4.0), and applied to a column (1.0 × 57 cm) of Sephadex G-50. Elution was carried out as described in MATERIALS AND METHODS. Peak A (tube Nos. 9–20) contained apurinic acid, and peak B (tube Nos. 24–38) contained only adenine and guanine as UV-absorbing materials.

activity of the phosphatase-treated material was not adsorbed on the column, as shown in Fig. 4b.

Depurination

The result of depurination of ^{14}C-associated DNA showed that the radioactivity per unit absorbancy at 260 nm in the dialysate was constant throughout the reaction (Fig. 5). Paper chromatography confirmed that the dialysate contained only purine bases as ultraviolet light-absorbing materials. The materials inside the cellophane bag were applied to a column of Sephadex G-50 and eluted with an acidic buffer. Apurinic acid was eluted as peak A and a small amount of purine bases which were left in the cellophane bag, were more slowly eluted as peak B (Fig. 6). 12% of the generated purine bases remained inside the cellophane bag, together with apurinic acid. The specific radioactivity of apurinic acid was 9% of the free bases in the dialysate. These results suggest that ^{14}C-labelled quinoline was associated with purine rather than the pyrimidine bases of DNA.

DISCUSSION

After a single intraperitoneal injection of labeled 4NQO into mice bearing Ehrlich carcinoma cells, considerable radioactivity was bound to DNA extracted from the ascites cells. The specific radioactivit yof the DNA reached a maximum 4 h after the injection and thereafter decreased gradually. Dilution by newly replicated DNA might explain this apparent decrease of the specific activity, since the amount of injected 4NQO was too small to inhibit the growth of carcinoma cells. SZAFARZ

AND WEISBURGER[15] studied the stability of the binding of radioactive metabolites of the carcinogen *N*-hydroxy-*N*-2-fluorenylacetamide to DNA in rat liver and found that there were two kinds of carcinogen–DNA binding, one of which was susceptible to enzymatic repair, while another was more resistant. EPSTEIN *et al.*[16] also reported the persistence of the binding of a carcinogen *N*-2-fluorenylacetamide or its metabolite to DNA in hyperplastic nodules of rat liver. In the case of 4NQO, the possibility of enzymatic removal of nucleotides modified by quinoline derivatives cannot be ruled out.

4NQO or its metabolites were previously demonstrated to be bound to DNA *in vivo*. The evidence for the binding obtained by earlier workers was that fluorescence was observed in DNA isolated from cells exposed to the carcinogen. TADA *et al.*[11] reported that the fluorescent compound was released from DNA as an alcohol-soluble material when the DNA was heat-denatured, whereas experiments by MATSUSHIMA *et al.*[10] in our laboratory demonstrated that the fluorescence still remained on the single-stranded DNA. The present investigation using ^{14}C-labeled 4NQO was in agreement with the latter finding. In the experiments described here, the filter-paper-disk method was adopted for measuring the acid-insoluble radioactivity. Heat or alkaline denatured DNA remained on the paper disk after successive washings with trichloroacetic acid, ethyl alcohol and ethyl ether. Free quinoline compounds intercalating in base pairs of DNA should be released from the disks. In fact, the radioactivity of [^{14}C]4NQO mixed with DNA, did not remain on the paper disk after these washings.

On the gel filtration and the DEAE-cellulose column chromatography, treatment with alkaline phosphatase from *E. coli* at 1 μg/ml did not change the position of the hydrolysate of ^{14}C-associated DNA which had been treated with pancreatic deoxyribonuclease plus snake venom phosphodiesterase, although deoxyribonucleotides were dephosphorylated. Digestion with much higher concentration of alkaline phosphatase resulted in the formation of a labeled compound which was more rapidly eluted than deoxyribonucleotides on gel filtration and which was not adsorbed on a DEAE-cellulose column. The presence of the quinoline moiety bound to nucleotides makes them less susceptible to alkaline phosphatase. A similar resistance to *E. coli* alkaline phosphatase was reported in the case of 7-methylguanylic acid[17].

The experiment of depurination indicates that about 90 % of the quinoline compound associated with DNA was bound to purine bases of the DNA. A preliminary experiment involving *in vitro* incubation of Ehrlich cells previously labeled with [^{3}H]adenine and [^{14}C]4NQO revealed the existence of a compound with ^{3}H and ^{14}C radioactivity, which accumulated for about one-third of the total ^{14}C-radioactivity bound to purine bases. The molecular structure of the purine–quinoline complex is now under investigation.

4NQO is known to be metabolically converted to 4HAQO. The diacetyl derivative of 4HAQO was noted to yield readily a quinoline–DNA complex *in vitro*[5]. The mechanism of quinoline–DNA complex formation may be related to that proposed for binding of *N*-acetoxy-*N*-2-fluorenylacetamide with the guanine moiety of DNA[18].

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INHIBITORY ACTION OF THYMINE RIBONUCLEOSIDE AND OTHER PYRIMIDINE DERIVATIVES ON THE CLEAVAGE OF 5-IODO-DEOXYURIDINE BY MOUSE-LIVER EXTRACT *

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SUMMARY

(1) The action of various pyrimidine derivatives on the enzymatic phosphorolysis of [^{125}I]5-iodo-2′-deoxyuridine ([^{125}I]IUdR) by a particulate-free fraction (100 000 × *g*) derived from mouse liver has been examined.

(2) The most efficient inhibitor of phosphorolysis investigated was thymine riþofuranoside (TR), the ribose analogue of thymidine (TdR), which is not itself cleaved by the enzyme extract.

(3) Although the apparent affinities of IUdR and TdR for the enzyme system were comparable, the maximum velocity of cleavage of the IUdR was approximately 5 times greater. This difference could explain the observed shorter duration of the homologue in the cell pool *in vivo* compared with TdR.

(4) The competitive inhibitory action of thymine ribonucleoside is approximately 20 times less effective for IUdR cleavage than for TdR cleavage by the enzyme extract. This difference offers a probable explanation for the 100-fold molar excess of thymine ribonucleoside necessary to inhibit loss of [^{125}I]IUdR from the cell pool *in vivo*.

(5) An extract from rat liver contained 5 times greater IUdR cleaving capacity than an equivalent extract of mouse liver.

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Abbreviations: IU, 5-iodouracil; IUdR, 5-iodo-2′-deoxyuridine; TdR, thymidine; TR, thymine ribofuranoside.

INTRODUCTION

IUdR is incorporated into deoxyribonucleic acid in place of TdR[1,2] but the extent of incorporation *in vivo* is limited, in part, by the catabolic activity of pyrimidine nucleoside phosphorylase. A preliminary report[3,4] described the inhibitory effect of TR on the enzyme cleavage of IUdR by an extract of mouse liver; subsequently, the effect of TR on the utilization of IUdR by the mouse[5–7] and the spleen cells of the golden-mantled ground squirrel *(Citellus lateralis)*[8] have been reported. A number of potential inhibitors of the cleavage of IUdR by an enzyme present in the soluble fraction derived from mouse liver have also been studied[9].

It is reasonable to expect that the enzymes in a soluble tissue extract that cleave IUdR would be related, if not identical to those normally present which cleave TdR. Evidence was obtained[10,11] for the existence of at least two enzymes, uridine phosphorylase and thymidine phosphorylase, both showing ability to cleave TdR but nevertheless differentially inhibited by deoxyglucosyl thymine. Further purification of these enzymes[12–14] resulted in the recognition of two enzymes with absolute substrate specificities for deoxyribosyl and ribosyl groups from *Escherichia coli* but which do not appear to be specific from mammalian sources. Whereas thymidine phosphorylase (thymidine:orthophosphate deoxyribosyltransferase, EC 2.4.2.4) has a cleavage specificity for deoxyribosylpyrimidines, uridine phosphorylase (uridine orthophosphate ribosyltransferase, EC 2.4.2.3) will cleave uridine, deoxyuridine, deoxythymidine and possibly uric acid-3-ribonucleoside[15]. The proportion of the two enzymes differ amongst species and this fact was utilized in demonstrating that deoxyglucosylthymine, which specifically inhibits uridine phosphorylase, the only pyrimidine nucleoside phosphorylase present in cat tissues, enhanced the incorporation of IUdR into the DNA of this species[16]. It is now recognised[17] that both uridine and thymidine phosphorylase exhibit pentosyl transfer either directly from one nucleoside to a pyrimidine or indirectly *via* pentose 1-phosphate.

We have reported[5] that [^{125}I]IUdR is incorporated less efficiently than TdR into the DNA of mouse tissues after parenteral administration. However, due to the more rapid clearance of the former nucleoside from the acid-soluble pool and the greater efficiency with which [^{125}I] may be measured, there are significant advantages in the use of this labelled precursor as a label of DNA *in vivo*, which has also been stressed by others[18,19]. Furthermore, the significant absence of re-utilization phenomena gives this labelled precursor an advantage over TdR as a short-term label for DNA in whole animals. This has been utilized in problems concerning cell proliferation and death[19], studies of bone marrow kinetics[20] and hydrocortisone effects on DNA synthesis of cultured Detroit cells[21]. The DNA so labelled appears to be biologically stable. The decreased efficiency of uptake of IUdR as compared to TdR into DNA *in vivo* has been partly explained[22] by the lowered affinity of the nucleoside for the thymidine kinase enzyme complex. The increased rate of removal of IUdR from the cell pool, however[5], is not explained by this mechanism and hence it appeared to us worthwhile to investigate these catabolic aspects in greater detail. The factors necessary for an irreversible inhibition of thymidine phosphorylase have been exten-

sively examined[23–25], and the relative affinities of arsenate and phosphate for the two enzymes have been compared[17]. The exaggerated nucleoside phosphorylase activity of mycoplasma-contaminated HeLa cells was inhibited by 5-allyl-2′-deoxyuridine[26]. Attempts[27] to increase the carcinostatic efficiency of 5-fluorodeoxyuridine by inhibiting the catabolic pyrimidine nucleoside phosphorylase failed.

We have now investigated the inhibitory action of TR for IUdR and TdR cleavage in further detail to attempt to give some explanation for the very large excess (100-fold molar)[7] of inhibitor required to be given *in vivo* to effect any significantly increased incorporation into DNA.

METHODS

Materials

3 enzyme preparations were used in this study. Preparation I was prepared from Swiss mice by homogenization of the liver with 4 volumes of 0.25 *M* sucrose at 0° and subsequent centrifugation at 100 000 × *g* at 4° for 1 h. Preparation II was prepared from female C3H mice by homogenization of the liver in 5 volumes 0.25 *M* sucrose at 0° in an all-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100 000 × *g* (Spinco, 40 rotor, 39 000 rev./min) for 30 min at 4°. The supernatant fluid (15.6 mg protein/ml) was diluted 6-fold with 0.25 *M* sucrose, divided into 5-ml volumes and stored at −20° until used. Preparation III was prepared from livers of rats of Wistar strain by the procedure described for Preparation II and stored in a similar manner.

Phosphate buffers were prepared from disodium hydrogen phosphate and sodium dihydrogen phosphate (0.2 *M*, pH 5.75). Tris–arsenate buffer was prepared by mixing 2 volumes of Tris–HCl buffer (0.5 *M*, pH 7.4) with 3 volumes of sodium arsenate (0.2 *M*, pH 7.4).

[^{125}I]IUdR was prepared by a modification of the method described previously[1]. A constant amount of [^{125}I]iodide was used and increments of deoxyuridine were added repeatedly until no further [^{125}I]IUdR crystallized from solution. The specific activity of [^{125}I]IUdR was the same for each crop of IUdR formed since the specific activity of the iodine remained constant. The specific activity of the product was 2.3 mCi/g (815 μCi/mole) and had less than 1% impurity when subjected to thin-layer chromatography on silica gel (see below).

We are grateful to Dr. P. K. CHANG who prepared TR by the procedure described by FOX *et al.*[28]. Other pyrimidine derivatives used in this study were kindly supplied by Dr. J. J. FOX of the Sloan-Kettering Institute, Dr. K. C. MURDOCK of the American Cyanamide Company and Dr. P. KENT of Oxford University.

The amount of protein present in the enzyme preparations was determined by the procedure of LOWRY *et al.*[29]. Crystalline bovine serum albumin (N.B.C., lot 3974) was used as standard.

Enzymatic methods

The following procedure was adopted in the preliminary studies of the inhibition

of the cleavage of [^{125}I]IUdR by a liver extract derived from Swiss mice (Preparation I). The reaction mixture consisted of [^{125}I]UdR (0.2 ml, 5 μmoles/ml), phosphate buffer (0.1 ml, 0.2 *M*, pH 5.75) and Enzyme Preparation I (0.1 ml). The final concentration of phosphate ion was 0.05 *M*. The inhibitor (5–10 μmoles) was dissolved in 1 ml of the phosphate buffer and 0.1 ml was added instead of the buffer to the reaction mixture. The reaction mixture was incubated at 37° for 10 min and enzymatic cleavage terminated by the addition of 0.4 ml trichloracetic acid (20%). The precipitate was removed by centrifugation and the supernatant fraction was analyzed by thin-layer chromatography on CM-cellulose as the solid layer and 0.23 *N* formic acid as the developing solvent. The separation was complete within 40 min at room temperature and the R_F value obtained for IUdR was 0.85 and for IU 0.60. Appropriate areas of cellulose were removed and assayed for radioactivity in a well-type gamma scintillation counter.

Both the phosphorolytic (pH 5.75) and arsenolytic cleavage (pH 7.4) of [^{125}I]UdR were investigated. The composition of the reaction mixture consisted of the substrate [^{125}I]IUdR (0.2 ml, 0.01–1.0 μmole/ml in the appropriate buffer), the buffer or a solution of TR in the buffer (0.2 ml) and Enzyme Preparation II or III (0.2 ml). The final concentration of phosphate or arsenate ion was 0.15 *M*. Duplicate reaction mixtures were incubated in a mechanically agitated water bath at 37°. After appropriate time intervals, the reaction mixtures were chilled in an ice bath and trichloracetic acid (0.2 ml, 20%) was added. The precipitate was removed by centrifugation at 3000$\times g$ for 5 min and the supernatant fraction was stored at −20° until analysed by thin-layer chromatography. In these studies an improved method of separation was used; Silica gel Hf was the absorbant and isopropyl alcohol:chloroform (20:80) the developing solvent. The R_F values* for IUdR is 0.4 and for IU, 0.7.

Regression lines, intercepts, and standard error data were obtained using a standard computer programme.

RESULTS

General inhibitor studies

The optimal pH for the cleavage of IUdR in phosphate buffer by Enzyme Preparation I derived from mouse liver is between pH 5.50 and 6.0 at a final ionic strength of phosphate of 0.05 *M*. Table I shows the extent of inhibition by various pyrimidine derivatives of the phosphorolytic cleavage of [^{125}I]IUdR by this enzyme. TR was the most effective inhibitor.

Kinetic studies

Enzyme Preparations II and III were used for the study of the reaction kinetics. The rates of arsenolysis (pH 7.4) rather than phosphorolysis (pH 5.75) were measured to reduce the reverse reaction of thymine or IU to the corresponding nucleoside[30] and to investigate the extent of the reaction under physiological conditions of pH. The final concentration of the arsenate ion in these studies was 0.15 *M*.

* The R_F values quoted in ref. 7 were inadvertently reversed.

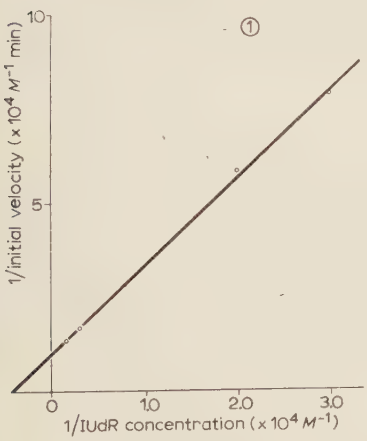
TABLE I

EFFECT OF PYRIMIDINE DERIVATIVES ON THE PHOSPHOROLYTIC CLEAVAGE OF [^{125}I]IUdR AT pH 5.75 FOR 10 min AT 37°
Final concentrations of [^{125}I]IUdR and inhibitor are 2.5 *M*.

Compound	*Inhibition* (%)	*Compound*	*Inhibition* (%)
Iodouracil	40	1-Cyclopentenethymine	10
Deoxyuridine	12	1-*n*-Butylthymine	4
Iodouridine	35	1-Phenylthymine	13
Thymine	46	1-(*cis*-3-Hydroxycyclopentyl)-thymine	2
Thymidine	40	1-(*cis*-3′,5′-Dihydroxycyclopentyl)thymine	12
Thymine ribofuranonucleoside	60	Arabinofuranosylthymine	3
5-Fluorodeoxyuridine	35	Xylofuranosylthymine	1
5-Iododeoxycytidine	—7	Xylopyranosylthymine	—8
5-Fluorouracil	2	Glucopyranosylthymine	—3
Azathymine	—10	Uracil	11
Azathymidine	—5	5-Hydroxymethyluracil	3
5-Hydroxyuridine	12		

IUdR cleavage

Using Enzyme Preparation II at a final concentration of 0.82 mg/ml, a double reciprocal plot (Fig. 1) gave values for the Michaelis constant (K_m) of 2.3 ($\pm$ 0.3)·10^{-4} *M* and a maximal velocity constant (V_m) of 0.10 ($\pm$ 0.01)·10^{-4} *M*/min. Under the same experimental conditions but using Preparation III from rat liver, at a final protein concentration of 0.16 mg/ml, the reciprocal plot of IUdR cleavage (Fig. 2) gave a K_m of 1.25 ($\pm$ 0.25)·10^{-4} *M* and V_m of 0.12 ($\pm$ 0.02)·10^{-4} *M*/min. The IUdR-cleaving activity of each enzyme preparation can thus be expressed as 190 U/ml (Preparation



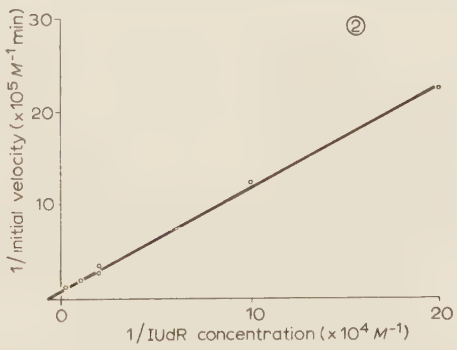


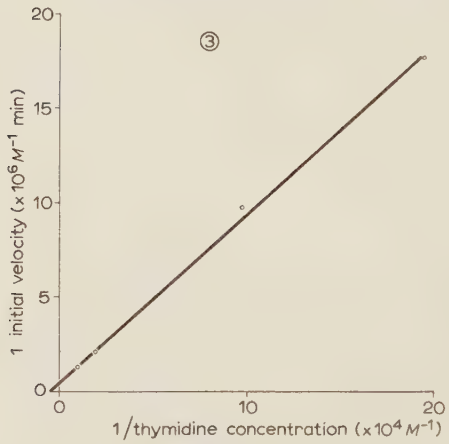
Fig. 1. Lineweaver–Burke plot of the arsenolytic cleavage of IUdR by Enzyme Preparation II. Final enzyme concentration 0.87 mg protein per ml incubation mixture. K_m = 2.33 ($\pm$ 0.30)·10^{-4} *M*, V_m = 0.10 ($\pm$ 0.01)·10^{-4} *M*/min.

Fig. 2. Lineweaver–Burke plot of the arsenolytic cleavage of IUdR by Enzyme Preparation III. Final enzyme concentration, 0.16 mg protein per ml incubation mixture. K_m = 1.25 ($\pm$ 0.25)·10^{-4} *M*, V_m = 0.12 ($\pm$ 0.02)·10^{-4} *M*/min.

II), 1200 U/ml (Preparation III). (1 unit is defined as the number of μmoles of substrate arsenolytically cleaved in 1 min at 37°, pH 7.4.)

TdR cleavage

A reciprocal plot was made of the arsenolytic cleavage of TdR by Enzyme Preparation II (Fig. 3), under the same conditions as with IUdR, and a K_m of 1.8 ($\pm$ 1.1)·10^{-4} M and a V_m of 0.02 ($\pm$ 0.01)·10^{-4} M/min were obtained. The reaction was noticeably slower and the values obtained for the maximal velocities suggest that about 5 times as much IUdR as TdR is cleaved at enzyme saturation per unit time. In terms of TdR cleaving units, the Preparation II was shown to be 38 U/ml.



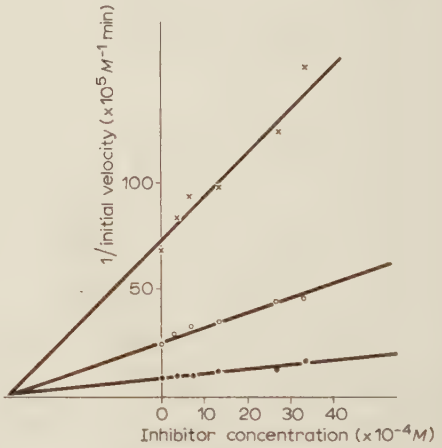


Fig. 3. Lineweaver–Burke plot of the arsenolytic cleavage of TdR by Enzyme Preparation II. K_m = 1.8 ($\pm$ 1.1)·10^{-4} M, V_m = 0.020 ($\pm$ 0.01)·10^{-4} M/min.

Fig. 4. Dixon plot of IUdR arsenolytic cleavage by Enzyme Preparation II. In the presence of the inhibitor ribofuranoside. K_i calculated from the negative intercept = 36·10^{-4} M. IUdR concentration ●, 0.31·10^{-4} M; ○, 0.084·10^{-4} M; ×, 0.031·10^{-4} M.

Inhibitory action of thymine ribonucleoside

TR inhibited the cleavage of IUdR by Enzyme Preparation II. A Lineweaver–Burke plot confirmed that the inhibition was competitive; a Dixon plot of $1/v_i$ against the concentration of inhibitor[31] (Fig. 4) gave an apparent K_i = 36·10^{-4} M and V_m 0.13·10^{-4} M/min. TR at a concentration range of 0.25–2.0·10^{-4} M inhibited the cleavage of TdR. A 'Dixon' plot (Fig. 5) of $1/v_i$ against I (concentration of inhibitor) gave an intercept at $1/v_i = 1/V_m$ = 40·10^4 M^{-1} min of K_i = 1.2·10^{-4} M. Cleavage of TR could *not* be detected under the conditions of arsenolysis with Enzyme Preparation II.

DISCUSSION

The extent to which IUdR is incorporated into DNA as an analogue of TdR will depend on its relative success as a substrate for catabolic and anabolic enzymes in competition with TdR. The relative competition with anabolic enzymes have been

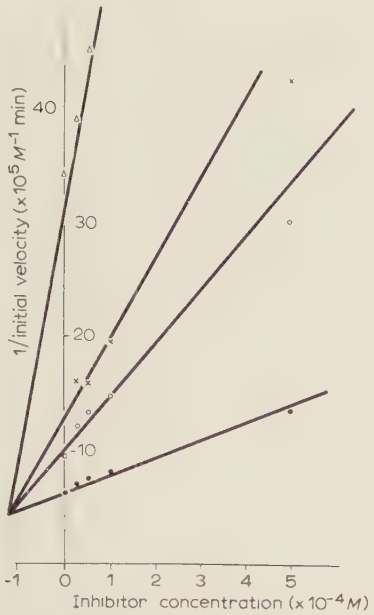


Fig. 5. Dixon plot of the arsenolytic cleavage of TdR by Enzyme Preparation II in the presence of the inhibitor TR. $K_i = 1.2 \cdot 10^{-4}$ *M*. TdR concentrations: ●, $2.0 \cdot 10^{-4}$ *M*; ○, $1.0 \cdot 10^{-4}$ *M*; ×, $0.50 \cdot 10^{-4}$ *M*; △, $0.25 \cdot 10^{-4}$ *M*.

studied[22] in rat-tissue extracts, but the contribution of the catabolic enzyme system is not known. Furthermore, the potential value of a successful inhibitor of this system in increasing the level of incorporation of homologues into DNA, cannot be underestimated. Of the many inhibitors of pyrimidine nucleoside phosphorylase investigated *in vitro* (pH 5.75) the analogue TR is one of the most effective[4]. This ribonucleoside is normally present in tRNA in an apparently non-random and hence potentially important role[32] and is formed by methylation of the uracil moiety within the polymer.

The kinetics of this crude enzyme system, composed of the 100 000 × *g* supernatant fraction derived from the homogenates of either mouse or rat liver, were examined by a standard assay procedure. 3 enzyme preparations were used, one for the general inhibitor studies, designed to screen a number of potential inhibitors, where only a relative comparison among a series of agents was examined. The other two preparations were used for more detailed kinetic studies. These latter were conducted at physiological pH 7.4 instead of 5.75 which has been shown to be the optimal condition for the phosphorolytic cleavage of TdR. Arsenolysis, rather than phosphorolysis was also used to simulate more closely the effect of product inactivation that would occur in whole tissue by loss through the cell membrane *in vivo*. There is now some evidence[17] that this latter choice may not be as reasonable as was originally thought, as the relative arsenate and phosphate affinities for the two enzymes appear to differ. However, since the data were obtained under constant arsenate concentration, the relative differences observed are considered to be valid.

Although the preparations used were crude, sufficiently consistent data were obtained to show that marked differences existed in the rate of cleavage of TdR and IUdR.

Although the apparent K_m of the enzyme for IUdR appears to be less than for TdR, the maximum velocity of cleavage of the natural nucleoside is approximately 5 times less. This greater rate of catabolism of IUdR in the presence of TdR would therefore account at least in part, for the more rapid removal of the IUdR from the mixture present in the cell pool. Since the non-polymerized phosphorylated derivatives are in equilibrium with these nucleosides, the different catabolic rate will be reflected in the composition of the whole cell pool. The lower affinity of IUdR for the TdR kinase system[22] would also assist this catabolic process. This mechanism would thus explain the recognized advantage of isotopically labelled IUdR as a 'pulse' label for DNA *in vivo* since its presence as a nucleoside within the cell would be of much shorter duration than that of the naturally occurring nucleoside.

A comparison of the ability of Enzyme Preparations II and III to cleave IUdR indicated that the preparation from rat liver is more than 5 times as active per unit of protein, even though it possesses kinetic properties comparable to that derived from mouse liver. The affinity of IUdR and TdR as substrates for their arsenolytic cleavage by the mixed enzyme system used were comparable (apparent K_m (IUdR)/ K_m(TdR) = 1.3). However, the turnover rate of IUdR to cleaved product was about 5 times greater than for TdR which would therefore explain the more rapid pool clearance reported *in vivo*.

The inhibitory action of thymine ribonucleoside against IUdR was considerably less efficient than anticipated in comparison with TdR and, therefore, its lack of acion *in vivo*[7] is not wholly unexpected. The apparent affinity of the inhibitor for the enzyme system was competitive and about 14 times less effective than IUdR which could explain the necessity of increasing the molar ratio *in vivo* to 100-fold before any significant accumulation of IUdR occurred within the acid-soluble pool.

The marked difference observed in the K_i of thymine ribonucleoside when determined in the presence of TdR ($K_i = 1.2 \cdot 10^{-4}$ M) compared to IUdR ($K_i = 36 \cdot 10^{-4}$ M) may be related to subtle differences in the K_m of the two substrates that are not apparent when comparison of average K_m values are made. The other possibility is that there are two enzymes involved, each having a different K value with respect to thymine ribonucleoside. The magnitude of the differences in K_i (30-fold) precludes an explanation for such differences in K_i, being a result of an interaction of substrate and inhibitor.

Thymine ribonucleoside, as well as a derivative of this compound has been demonstrated recently to participate in several biologically important reactions. KESHGEGIAN *et al.*[33] reported that this compound inhibited markedly mitosis in normal human lymphocytes incubated *in vitro* with phytohaemagglutinin. The addition of TdR at a concentration of 5% that of thymine riboside reversed the inhibitory effect. The question of whether thymine riboside or a derivative of it has a role in normal physiological reactions, in addition to being a component of some RNA species is an intriguing one and remains to be explored.

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ORGAN CULTURE OF FETAL-RAT PANCREAS

III. ULTRASTRUCTURAL CHANGES OCCURRING IN B CELLS DURING STIMULATION OF INSULIN RELEASE*

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SUMMARY

Fetal-rat pancreas, explanted on the 18th day of gestation and cultured over 4 days, was used for the study of morphological changes occurring in B cells and associated with the stimulation of insulin release *in vitro* during a 2-h incubation period. This preparation is characterized by its ultrastructural preservation and by the fact that it can release up to one third of stored insulin when incubated in the presence of appropriate stimuli.

Under these conditions, dramatic morphological changes were observed in β-granules. They appeared widened and their core, losing its characteristically homogeneous structure, was progressively substituted by filamentous material, thus suggesting granular disintegration within the membranous envelope and presumably into the cytoplasm. In all instances, the membranous envelopes remained uninterrupted.

Whatever the stimulus, these ultrastructural changes were qualitatively similar but varied quantitatively with the intensity of the stimulation of insulin release.

These findings suggest that intracytoplasmic release of secretory material through intracellular solubilization of β-granules is the predominant mode of insulin secretion in cultured fetal explants.

Since there is also some evidence for "emiocytosis" in this preparation, it thus appears that insulin secretion may proceed from several (or at least from two) ultrastructural events according to the preparation and/or the stimulus used.

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INTRODUCTION

Granulation of pancreatic B cells increases parallel with insulin content and decreases after strong stimulation of insulin release. These relationships, first studied by HARTROFT AND WRENSHALL[1] clearly indicate that β-granules represent a storage form of insulin. Ultrastructural studies of insulin secretion have been mainly carried out *in vivo*. Since the reports of LACY *et al.*[2] and WILLIAMSON *et al.*[3], it has become generally accepted that the release of β-granules results from a process of "emiocytosis", *i.e.* the ejection of granules from B cells into the extracellular space. More recently, however, other mechanisms have been proposed. LEVER AND FINDLAY[4] have described the intracytoplasmic release of secretory material through a perforation of the membrane of β-granules. HAIST[5] and CREUTZFELDT[6] have speculated that intracellular solubilization of β-granules may occur by a non-specified mechanism. Moreover, according to these authors[5,6], insulin release might also result from the passage of a more soluble, non-granular, form of insulin from its site of synthesis directly to and through the plasma membrane.

Only a few studies have been published concerning ultrastructural changes related to insulin release *in vitro*, using rabbit-pancreas fragments[7–9] or isolated islets of Langerhans of the rat[10]. The results of these studies presented many contradictory features and did not lend predominant support to any one mechanism.

Since a different preparation, that of cultured fetal-rat pancreatic explants, has been extensively used in our laboratory as a model for the study of insulin biosynthesis and release, the present paper will describe the morphological changes occurring in the B cells of this preparation, when incubated *in vitro* in the presence of some stimuli of insulin release.

MATERIALS AND METHODS

The culture method used in our studies has already been described[11,12]. In summary, fetal-rat pancreas were explanted on the 18th day of gestation under sterile conditions. They were cultured over 4 days according to a modified watch-glass technique in a medium containing 90 % (v/v) Eagle HeLa medium, 5 % (v/v) adult-rat serum, 5 % (v/v) rat embryonic extract and penicillin G, 100 U/ml. The final glucose concentration of the culture medium was 16.5 m*M*.

Cultured explants for the study of insulin release were washed in a Krebs–Ringer bicarbonate buffer containing 2 % (w/v) human serum albumin. The ionic composition of this medium was, in m*M*: Na^+ 143.3, K^+ 6.0, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 126.0, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, and HCO_3^- 24.6. Unless the variables studied included changes in ionic composition, preincubation for 15 min and incubation for 120 min were subsequently carried out in the same Krebs–Ringer bicarbonate buffer. The agents to be tested for their effect on insulin release were added at the beginning of the incubation period. The effects of potassium were studied by altering the ionic composition of the incubation buffer during both preincubation and incubation as follows: KCl and KH_2PO_4 (replaced by NaCl and NaH_2PO_4) were omitted ($K^+ = 0$)

or KCl was added so that the final potassium concentration was 48 m*M*. The isotonicity of the medium was maintained by adjusting NaCl concentration.

In each instance, the cationic composition of the medium as well as its possible interference with the measurement of insulin were checked. All incubations were carried out at 37° with continuous shaking and in an atmosphere of 93–95 % O_2 and 5–7 % CO_2. Under these conditions, the pH of the medium remained at 7.4. At the end of the incubation period, the pancreatic explants were immediately fixed for subsequent morphological analysis or were blotted and weighed. The incubation media were frozen until assayed. The insulin release occurring during the incubation period was expressed in microunits per mg wet weight and over 2 h of incubation.

Insulin was measured according to the radioimmunoassay method of HALES AND RANDLE[13]. This method was slightly modified by using centrifugation instead of ultrafiltration for the separation of the antibody-bound insulin.

Explants for morphological analysis were fixed in glutaraldehyde (2.5 or 3 % solution in phosphate buffer), postfixed with OsO_4 according to MILLONIG[14] and embedded in Epon[15]. Semi-thin sections were first observed with the phase-contrast microscope. Contiguous thin sections were treated with lead hydroxide[16] and observed with the electron microscope (Zeiss EM 9 or Philips EM 300).

Chemicals and reagents. Eagle HeLa tissue culture medium was obtained from Difco Laboratories, Detroit (U.S.A.). Human-serum albumin was purchased from the Swiss Red Cross, Berne (Switzerland). D-Glucose and caffeine were obtained from Merck A.G., Darmstadt (Germany). Tolbutamide and glybenclamide (HB 419) were gifts from Farbwerke Hoechst A.G., Frankfurt/Main-Hoechst (Germany).

RESULTS

Insulin release

The addition of caffeine to the incubation medium stimulated somewhat the insulin release and remarkably enhanced tolbutamide-induced or glybenclamide-induced insulin release in the absence of any glucose (Fig. 1).

The omission of potassium from the Krebs–Ringer bicarbonate buffer stimulated insulin output, as did raising of the potassium concentration to 48 m*M* (Table I). Furthermore, the effect of the combined presence of glucose and caffeine was even more marked in a medium containing either no potassium or a high potassium concentration (Table I). Indeed, in the simultaneous presence of glucose, caffeine and an elevated potassium concentration, the explants released more than 1 milliunit of insulin per mg of wet tissue weight (*i.e.* about 30 % of the initial insulin content) during 2 h!

Ultrastructure of B cells in cultured explants

The conservation of fetal pancreas cultured over 4 days appeared excellent. In particular, all the cellular organelles including secretory granules are remarkably well preserved (Fig. 2). The fine structure of the islets of Langerhans in cultured

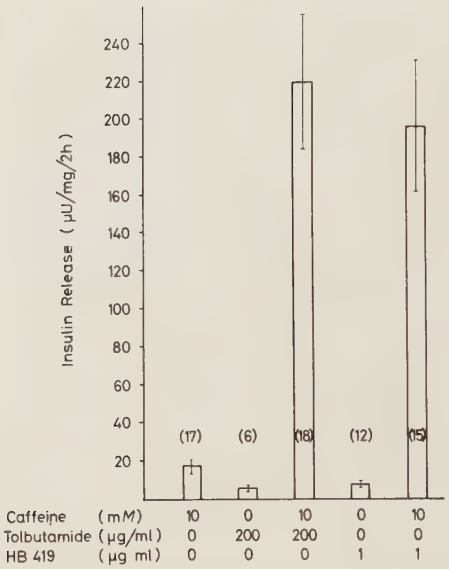


Fig. 1. Effect of tolbutamide or glybenclamide (HB 419) alone or together with caffeine on insulin release by fetal-rat pancreas previously cultured over 4 days. Each column represents the mean ± S.E.M. of the number of observations indicated between brackets.

TABLE I

EFFECT OF VARIATIONS OF POTASSIUM CONCENTRATION IN THE MEDIUM ON INSULIN RELEASE BY CULTURED EXPLANTS OF FETAL-RAT PANCREAS INCUBATED *in vitro*

Each figure represents the mean ± S.E.M. of the number of observations shown in brackets.

Additions	*Concentration of potassium*		
	0 mM	*6 mM*	*48 mM*
	Insulin release (μU/mg/2 h)		
None	22.3 ± 4.1 (11)	5.3 ± 1.4 (12)	52.3 ± 11.3 (12)
5.5 m*M* glucose + 10 m*M* caffeine	624.1 ± 41.5 (13)	239.8 ± 39.7 (10)	1254.9 ± 98.0 (13)

explants was generally similar to that described for adult-rat islets by LACY[17] and LACY *et al.*[2]. β-Granules were randomly distributed throughout the cytoplasm and their number varied considerably from cell to cell, some B cells containing rare secretory granules while others were filled with them. Typically, β-granules consisted of a smooth membranous sac separated from its content by a relatively large electron-lucent halo. There was considerable variation in the shape of the central material within β-granules. According to their electron-density, 2 main groups of secretory granules might be distinguished: pale and dense granules (Fig. 2). Both types of granules may be present within the same cell. However, many intermediate densities of the core existed between these two types and, in a few instances, β-granules appeared completely empty.

An important feature of the morphology of islets from cultured explants was

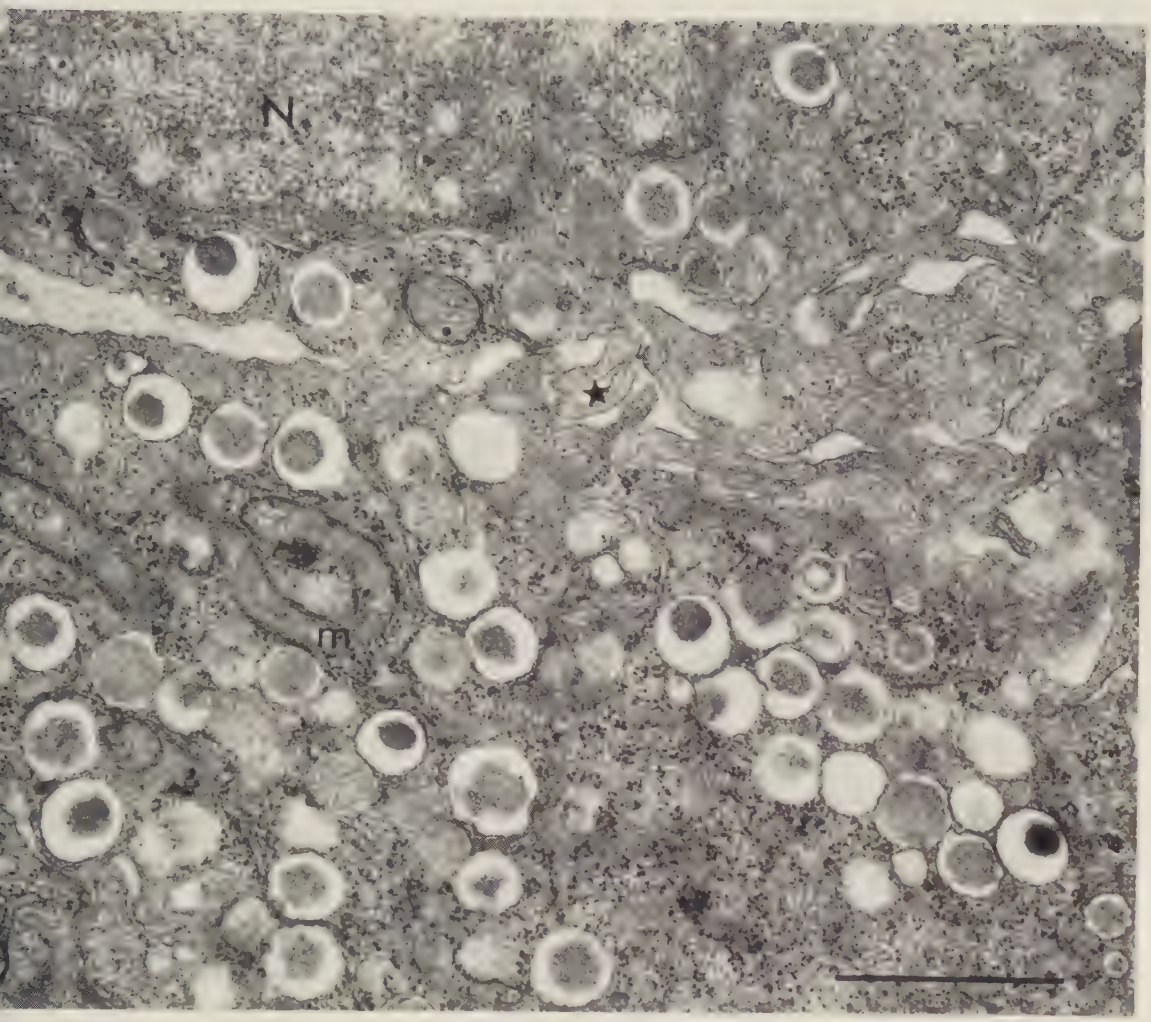
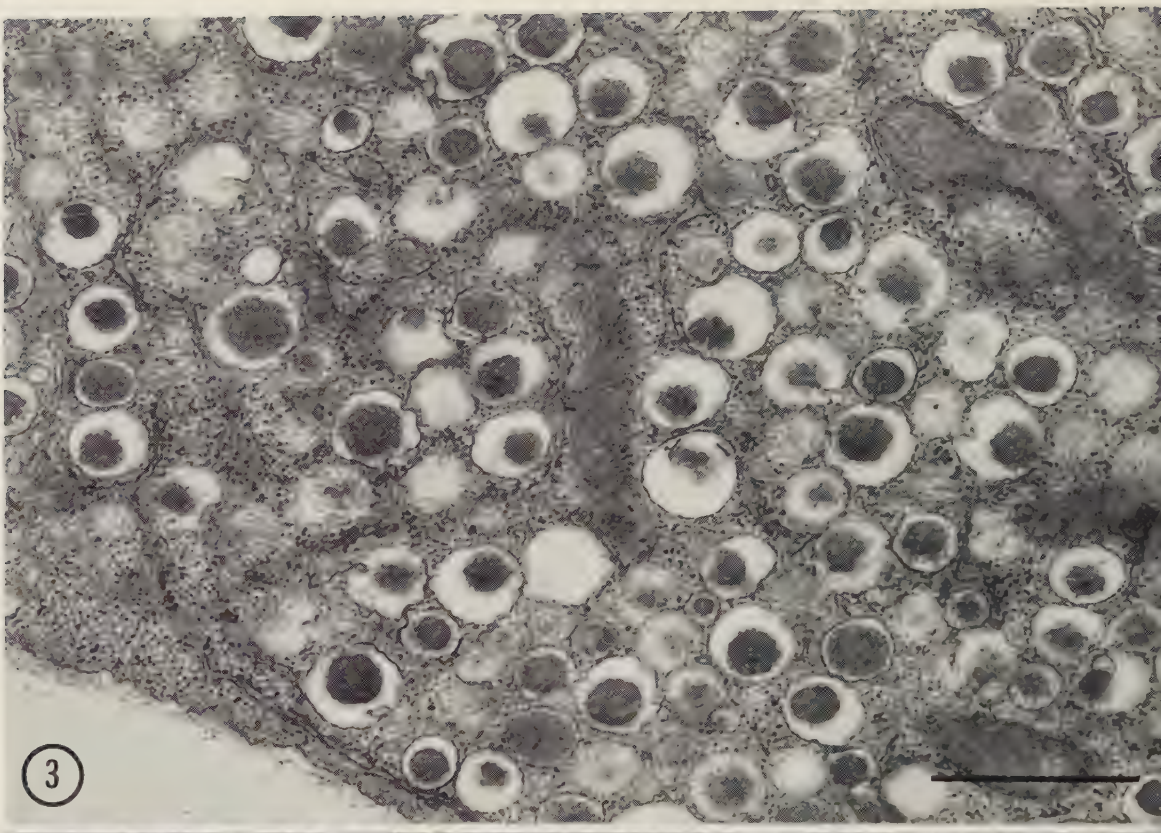


Fig. 2. Fetal-rat pancreas explanted on the 18th day of gestation and cultured over 4 days. Parts of B cells containing pale and dense secretory granules. Note the presence of intercellular space (*) with many finger-like processes bathing in it. N = nucleus; m = mitochondrion. × 30 500.

the presence of a relatively large extracellular compartment. In the intercellular space, finger-like processes may be seen (Fig. 2).

Ultrastructure of B cells in cultured explants after incubation

After 2 h of incubation *in vitro*, the general morphological appearance and conservation of our preparation appeared comparable to that of cultured explants before incubation. When incubated under basal conditions (normal Krebs-Ringer bicarbonate buffer) or in the presence of agents which are—under the conditions selected—poor stimulators of insulin release (caffeine alone; no or 48 m*M* potassium in the *absence* of glucose and/or caffeine), the ultrastructure of B cells and, in particular, of β-granules was essentially similar to that of non-incubated pancreas (Figs. 3 and 9).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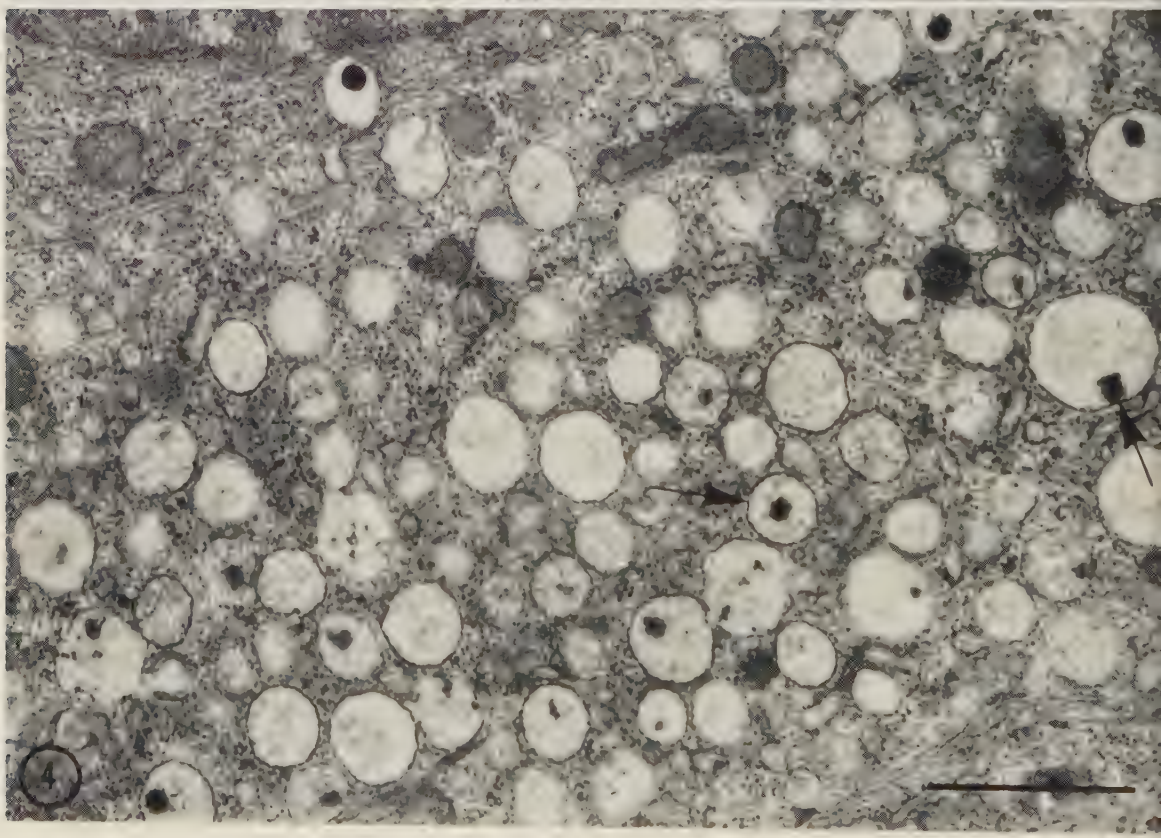
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By contrast, dramatic morphological changes were observed in β-granules when the cultured explants were incubated in the presence of potent stimulators of insulin release. The secretory granules showed many stages of granular dissolution which could be sequentially classified as follows: (*1*) Decreasing size of the dense material within the granule (Figs. 10 and 11). (*2*) Fragmentation of the core into electron-dense particles, some of them in close contact with the granule limiting membrane (Fig. 14). (*3*) Progressive loss of the characteristics of the core, substituted by filamentous and/or flaky material (Figs. 4 and 11–13). (*4*) β-Granules completely devoid of any electron-dense material and reduced to a membrane surrounding an electron-lucent area (Figs. 5 and 10).

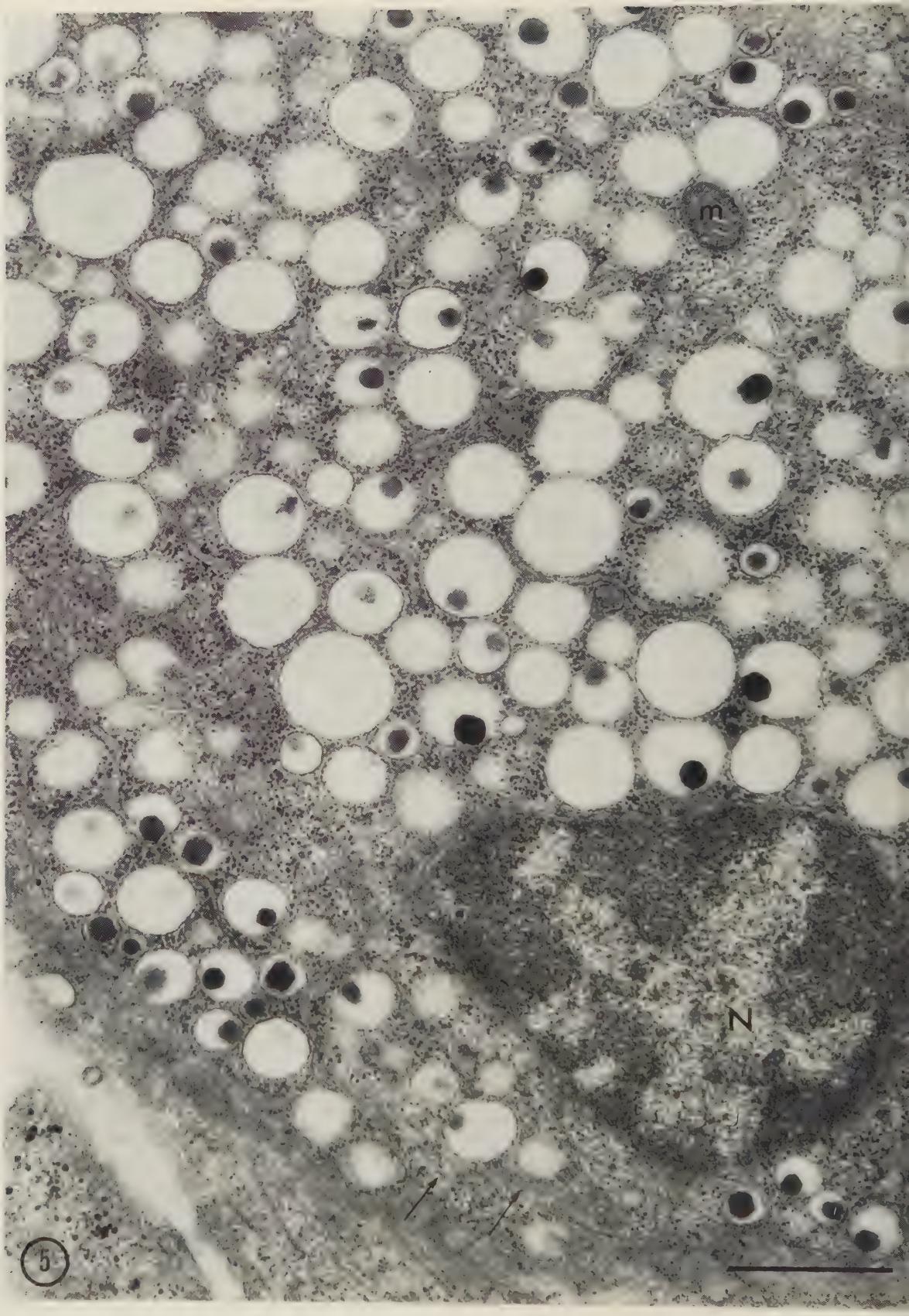
In all instances, the membrane of well-rounded, even distended secretory granules remained intact, without any evidence of perforation. However, some β-granules that were completely devoid of their content showed striking additional variations in their morphology. We would like to describe them as a sequence that might serve as basis for the discussion of a possible mechanism for disposal of the "emptied sacs": in addition to the rounded profiles of uninterrupted membranous sacs, others clearly show an invagination (Fig. 16) which may become deeper, giving the sac the appearance of a deflated punching ball, with the inner, infolded membrane lying closer to the outer, still circular membrane (Fig. 17). After this, the flattened membranous sac undergoes fractionation, producing profiles such as those seen in Figs. 18 and 19 where the crescent-shaped profile of two parallel membranes is disrupted into two or more fragments, leaving tubular and vesicular elements that still, by their position around a relatively electron-lucent area of granular size, recall the initial membranous sacs (Fig. 20). These morphological changes are schematically summarized in Fig. 21.

Other structures, though less frequent than those just described, suggest another possible mechanism for membranous sac disposal. Thus, one may see emptied granules with irregularly folded membranes which may fragment locally into two or several elements or pieces of "double membranes" sometimes resembling myelin figures. These figures may in turn conglomerate to form highly complex bodies containing densely packed membranous whirls in close approximation to circular electron-lucent areas of the size of the original membranous sacs (Figs. 22 and 23).

Whatever the agent(s) tested, the ultrastructural changes described were always present and qualitatively similar, provided the combination of agents was effective in producing major insulin release. They differed only in quantitative terms (*i.e.* the proportion of B cells within each islet and of β-granules within each cell

Fig. 3. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 48 m*M* potassium. B cell appears well preserved and contains typical β-granules. × 27 000.

Fig. 4. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 48 m*M* potassium, 5.5 m*M* glucose and 10 m*M* caffeine. In the centre of the Figure, part of one B cell. Most of the granular sacs contain flaky and filamentous material with, in some instances, remnants of core material (arrows). × 27 000.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showing these changes) according to the intensity of the stimulation. The most striking changes were those observed after incubation in the combined presence of glucose and caffeine in a medium also containing 48 m*M* K^+, when the majority of B cells showed granular dissolution. When incubated with glucose and caffeine in the absence of potassium, the ultrastructural changes of β-granules were relatively less numerous. They were again less marked, although clearly present, in explants incubated with sulfonylureas or glucose together with caffeine, yet at a normal potassium concentration.

In B cells of non-incubated explants as well as in those examined after incubation with a potent stimulator of insulin release, secretory granules were randomly distributed throughout the cytoplasm, although some were peripheral and sometimes showed margination. Extrusion of β-granules into the extracellular space was occasionally observed in our preparation.

An interesting additional finding, frequently seen in partially degranulated B cells, was the presence in the immediate vicinity of the Golgi complex of electron-lucent vesicles of a size intermediate between that of fully mature β-granules and that of smooth or coated microvesicles (Figs. 7 and 8).

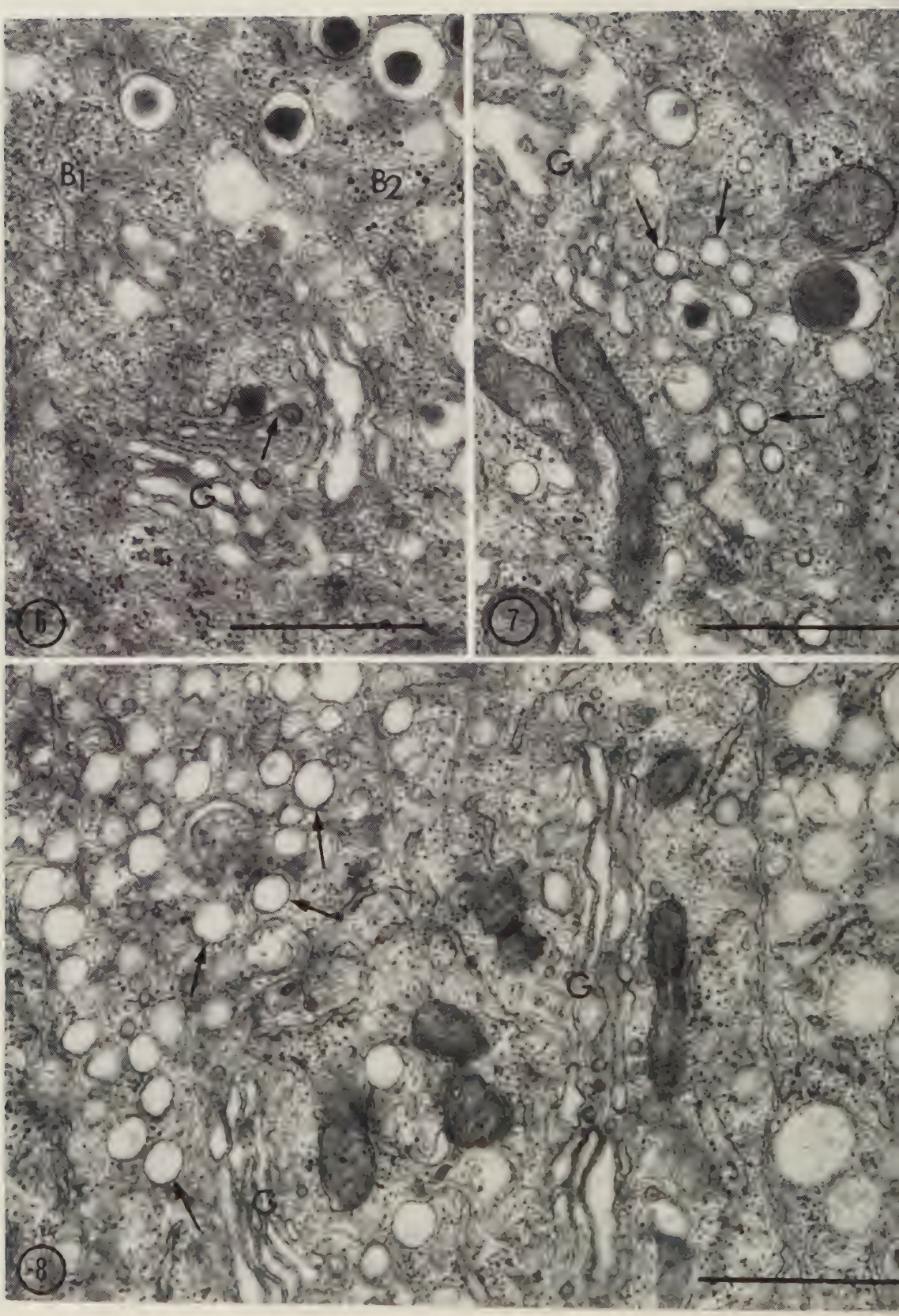
DISCUSSION

Insulin release

The regulation of insulin release from cultured fetal pancreas has been extensively studied in our laboratory, as reported elsewhere[11,12,18–21]. The main characteristics only of our preparation will be briefly summarized here: these are the relative ineffectiveness of glucose alone in promoting insulin release and the remarkable enhancing effect of caffeine on the effect of most stimulators, including glucose. The poor response of cultured explants to glucose might be related to the fetal nature of that preparation since ASPLUND *et al.*[22] have observed that, in the rat, the stimulatory effect of glucose on insulin secretion appeared only on the second day after birth. The effect of caffeine suggests that 3′,5′-cyclic AMP is *one* of the crucial intermediates needed for activating insulin release and we have postulated that phosphodiesterase might be hyperactive in our cultured fetal preparation[19].

A stimulatory effect of tolbutamide, either alone or in the presence of glucose, has been observed in all preparations of isolated pancreas studied so far. In cultured explants, however, the presence of glucose is required for good effectiveness and caffeine could replace glucose in this enhancing effect. The remarkable insulin output observed under such conditions, even in the complete absence of any added substrate, clearly demonstrates that, in our preparation, endogenous energy stores are sufficient for insulin release of considerable magnitude for at least 2 h.

Fig. 5. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 48 m*M* potassium, 5.5 m*M* glucose and 10 m*M* caffeine. Part of a B cell: most of the secretory granules appear empty. Only a few granules are normal. Note the presence of numerous ribosomes. The arrows point to microtubular profiles. m = mitochondrion; N = nucleus. × 27000.


B1
B2
G
6
G
7
G
G
8

GRODSKY AND BENNETT[23] and MILNER AND HALES[24] have first described the marked effects of some cations, in particular calcium and potassium, on insulin release. In cultured explants, either the absence or the presence of excess of potassium in the incubation medium stimulates insulin release. The unusually large insulin output observed in the combined presence of glucose, caffeine and a high potassium concentration, does not seem to result from "leakage" of insulin from damaged B cells since it is reversible[21]. On the other hand, the K^+-induced insulin release was not affected by lowering the sodium concentration in the medium[21]. The stimulation of insulin release observed in the absence of extracellular potassium might result from inhibition of the sodium pump with intracellular accumulation of sodium. In fact, ouabain (a well-known inhibitor of the sodium pump) enhanced the effect of glucose and caffeine in our preparation while lowering of the sodium concentration in the medium suppressed the stimulatory effect of the omission of potassium[21].

These results clearly establish the importance for insulin-releasing mechanisms of maintaining ionic balance between the extracellular and intracellular compartments. They further suggest that depolarization of the B cell membrane activates insulin output. When lowering the extracellular potassium concentration, the stimulatory event might be the accumulation of sodium within B cells while the extracellular accumulation of potassium might similarly provide the stimulatory event when increasing the potassium concentration in the medium. In any case, it seems likely that, in both instances, the final stimulatory event might be changed in the concentration and/or repartition of intracellular calcium, which also occurred during cell-membrane depolarization.

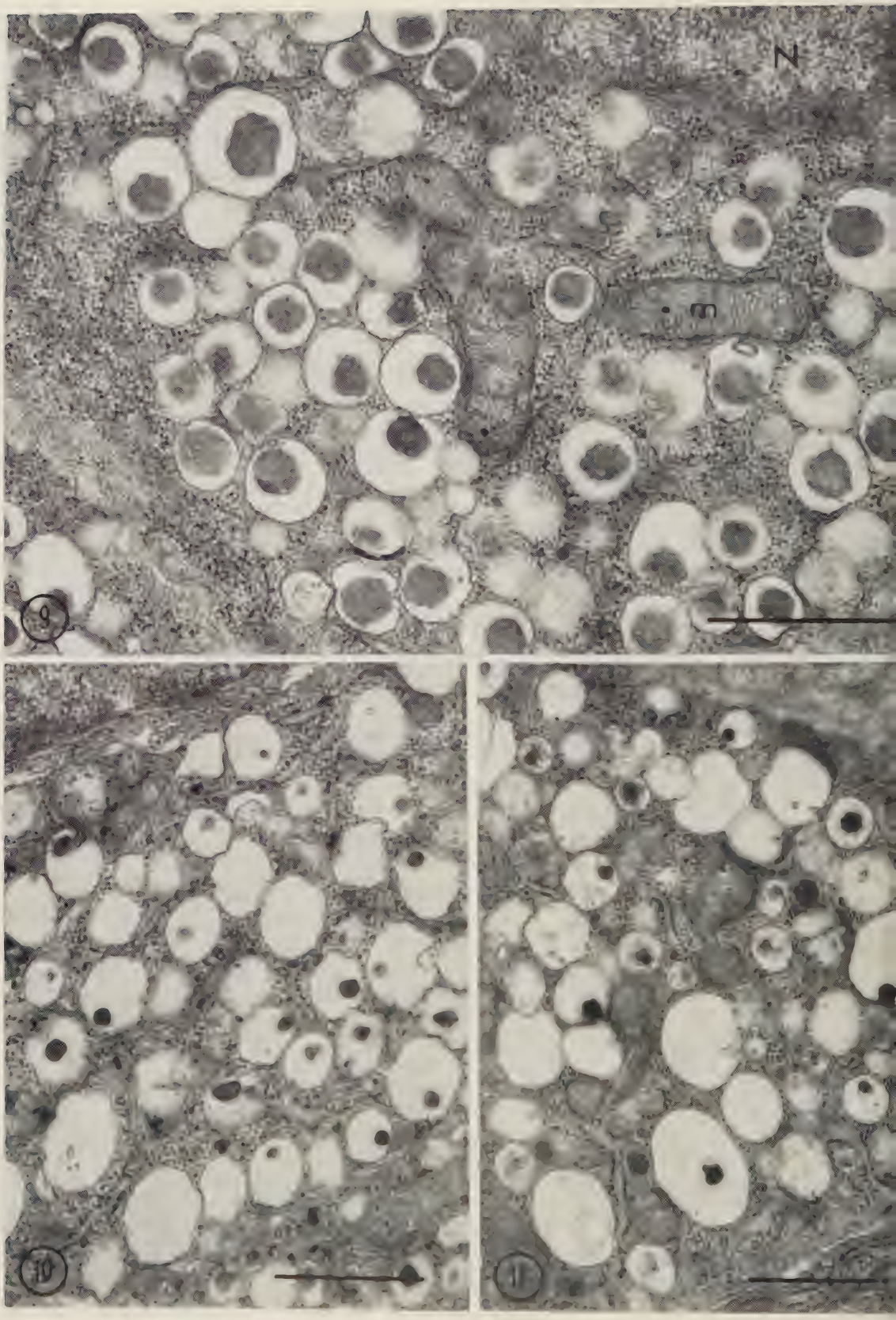
Morphological analysis of B cells

Previous studies dealing with the ultrastructural events associated with insulin release *in vitro* have been hampered by the relatively poor preservation of the preparations used[9,10] as well as by the fact that the maximal amount of insulin releasable by these preparations corresponded to only a few percent of the total insulin content. By contrast, our extensive studies with the electron microscope have shown that the preservation of cultured fetal pancreas was excellent up to 8–10 days of culture and entirely comparable to that of tissue maintained *in vivo* until fixation[25]. As compared to the adult, endocrine pancreas occupied a relatively larger proportion of the total organ and consisted predominantly of B cells, although some granulated

Fig. 6. Fetal-rat pancreas explanted on the 18th day of gestation and cultured over 4 days. Parts of 2 B cells. In B_1 one cisterna of the Golgi complex (G) contains a maturing secretory granule. The arrow indicates a direct communication between this cisterna and a coated microvesicle. × 33 000.

Fig. 7. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 5.5 m*M* glucose, 10 m*M* caffeine, but no potassium. Part of the B cell in the Golgi region (G) containing vesicles (arrows) having a size intermediate between that of microvesicles and that of mature secretory granules. × 33 000.

Fig. 8. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 48 m*M* potassium, 5.5 m*M* glucose and 10 m*M* caffeine. In the central part of the Figure, one B cell with the Golgi complex (G) surrounded by many electron-lucent vesicles (arrows). × 32 000.


N
9
10
11

A cells were also seen[26]. This ultrastructural integrity, together with the capacity to increase considerably the amount of stored insulin during the culture period[27] and with that to release a large fraction of stored insulin during a relatively short incubation period represent the main advantages of our preparation and prompted us to reinvestigate the ultrastructural aspects of insulin secretion *in vitro*.

The most striking change occurring in B cells of pancreatic explants incubated under conditions leading to stimulation of insulin release was the increase in "empty" sacs, together with modifications of the aspect and the electron-density of the core of β-granules, suggesting changes in the physico-chemical state of stored insulin which may reflect a granular disintegration. These changes are similar to, and even more marked, than those observed in pancreatic B cells *in vivo* following the administration of sulfonylureas in the rat[28] and give experimental support to the hypothesis of HAIST[5] and of CREUTZFELDT[6]. The process of intracytoplasmic solubilization of β-granules was not associated with the disruption of limiting membranes. We have never seen in our material the vesicular perforations reported on by LEVER AND FINDLAY[4] and FINDLAY *et al.*[9].

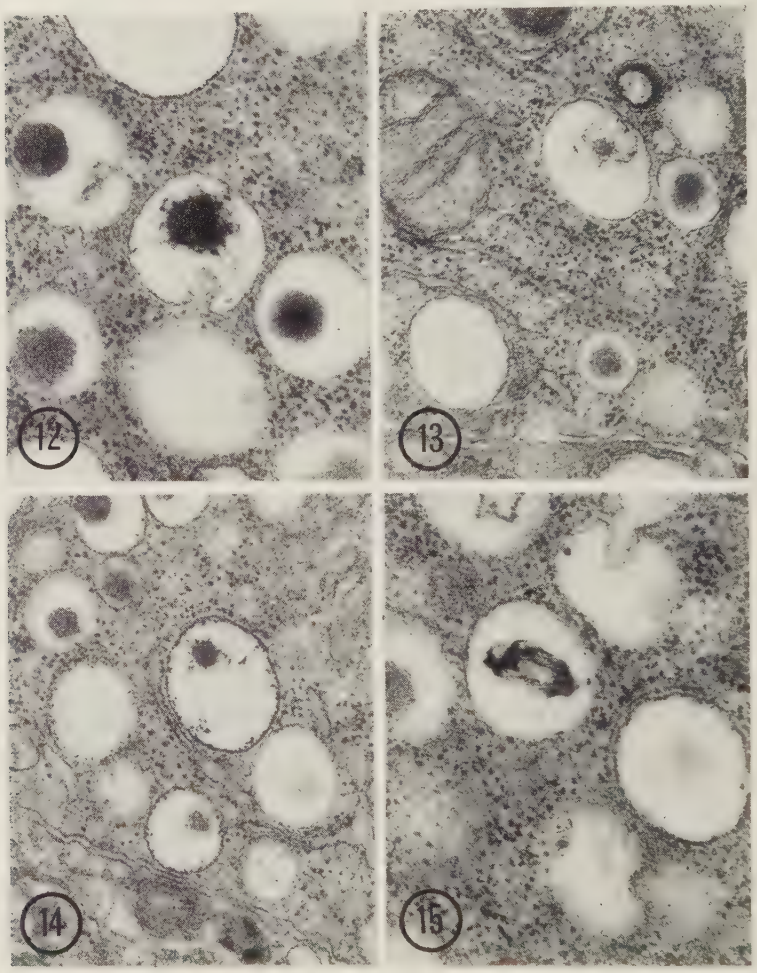
Of course, we are aware of the fact that an "empty" granule may still contain insulin. However, the parallelism between the afore-mentioned morphological changes and the intensity of insulin release suggests that these changes represent a morphological basis for insulin release in our preparation.

Since emiocytosis may be a very quick phenomenon, we cannot exclude it as a mechanism for insulin release in this preparation also. It would, however, seem logical to assume that emiocytosis is not a quantitatively major event capable of carrying the very large proportion of stored insulin released in some of our experiments. Moreover, insulin release by emiocytosis is said to involve the formation of cellular digitations and their increase is thought to express accelerated insulin release. Our observations in cultured explants, as well as *in vivo* in the rat[28], have clearly shown that the extracellular space in the endocrine pancreas is normally relatively large and characterized by numerous cellular digitations, even at rest. This is in agreement with the findings of MATSCHINSKY AND ELLERMAN[29] who have shown that the extracellular space normally represents 39% of tissue water in isolated islets of Langerhans. Although quantitative measurements are still lacking, it appears that neither the extracellular space nor the number of cellular digitations varied appreciably in our explants, whether or not insulin release was stimulated.

Fig. 9. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 10 m*M* caffeine. Parts of B cells containing secretory granules with similar characteristics to those shown in Figs. 1 and 2. m = mitochondrion; N = nucleus. × 32 000.

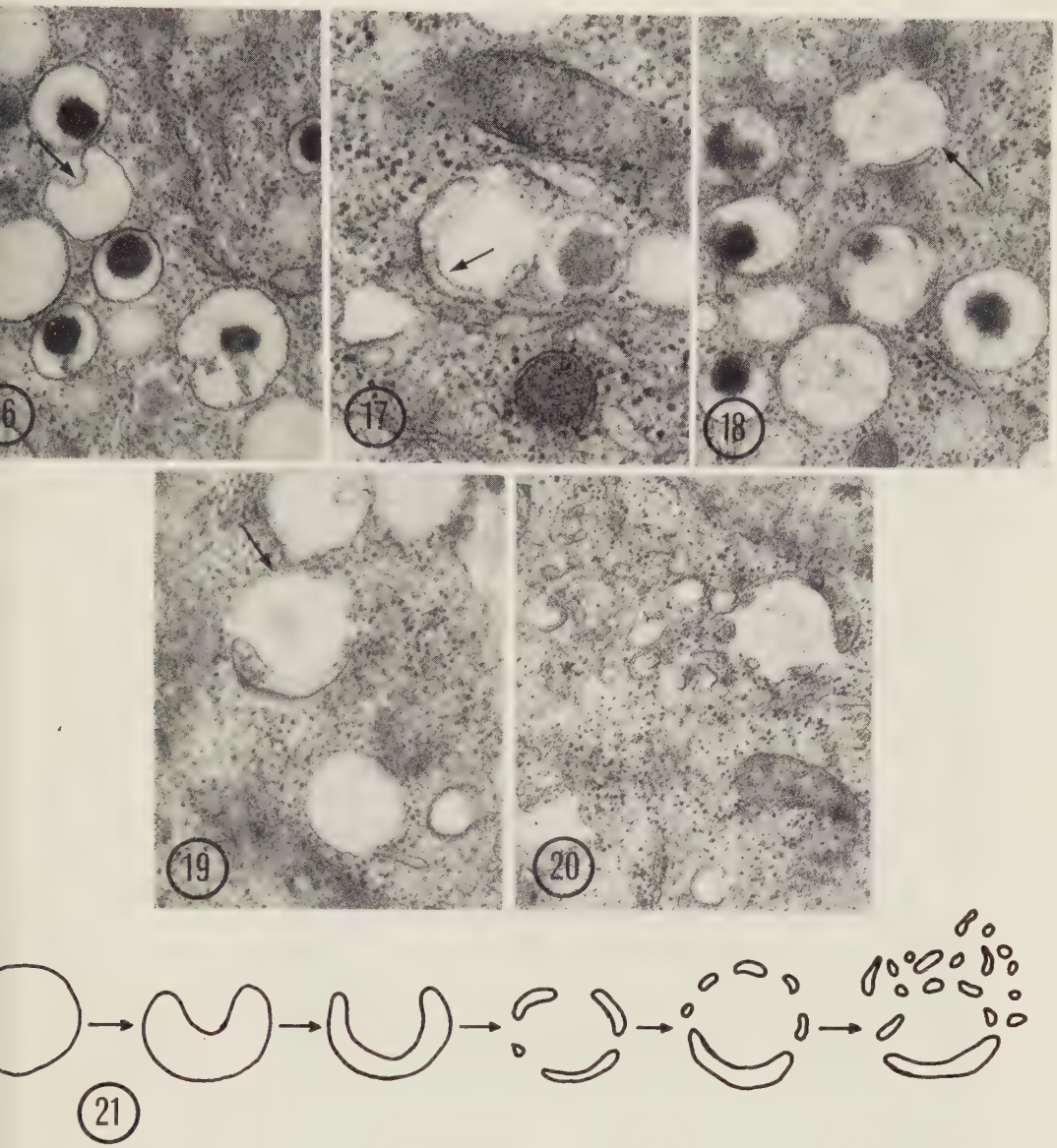
Fig. 10. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 10 m*M* caffeine and tolbutamide (200 μg/ml). Many β-granules appear empty while others contain only remnants of the central material. × 25 000.

Fig. 11. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 10 m*M* caffeine and glybenclamide (1 μg/ml). Most of the sacs contain flaky material. × 25 000.



Figs. 12–15. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 48 m*M* potassium, 5.5 m*M* glucose and 10 m*M* caffeine. Examples of alterations occurring in the central material of β-granules. × 30 000.

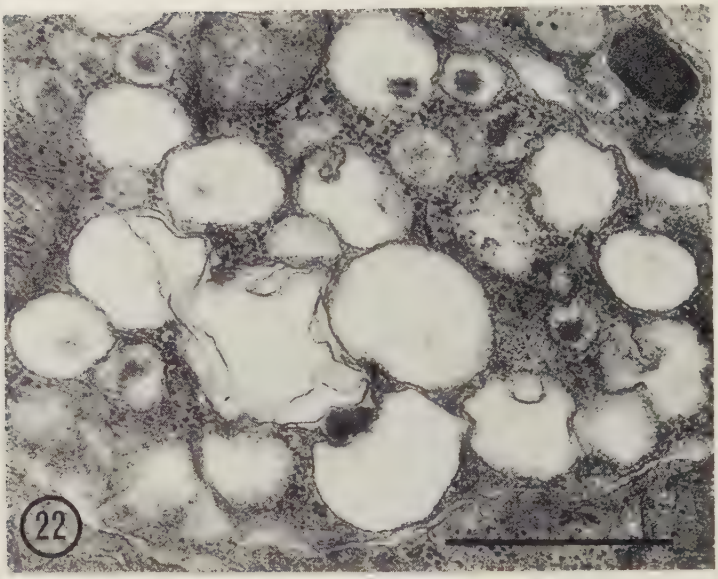
Acceptance of the intracellular dissolution of β-granules as another mechanism of insulin release implies a new hypothesis and requires new mechanisms for the protected transport of insulin through the cytoplasm and through the B cell membrane, and also for the disposal of the empty membranous sacs. WHALEY *et al.*[30] have proposed an attractive theory for the rapid and probably reversible formation of endoplasmic reticulum. According to these authors, this organelle is formed through the coalescence and the lamellar organization of molecular building blocks previously present in the soluble part of the cytoplasm. As the membranes seemingly disappear as rapidly as they appear, one might speculate that such a mechanism might be relevant to other cytoplasmic membranes, in particular to the disappearance of β-granule membranes by solubilization. Under RESULTS we have shown evidence for two possible processes, one leading to disappearance, the other to confluence

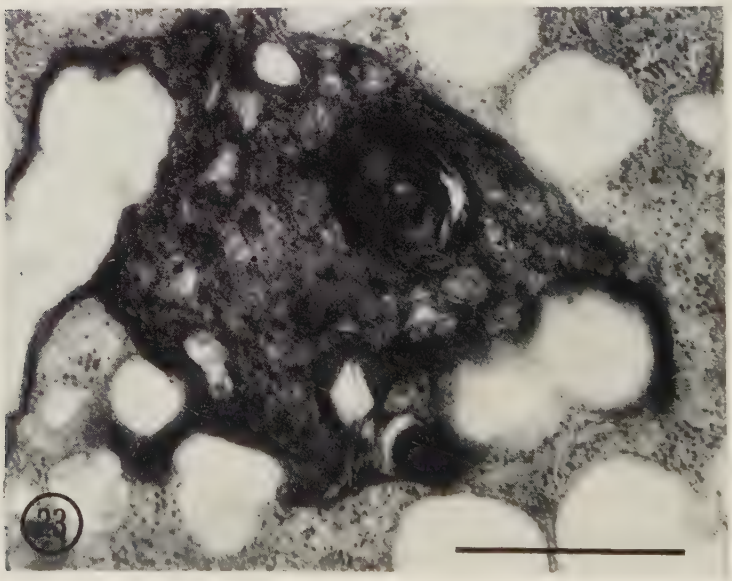


Figs. 16–21. Fetal-rat pancreas explained on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 48 m*M* potassium, 5.5 m*M* glucose and 10 m*M* caffeine. The arrows point to alterations occurring in the granule limiting membrane. A hypothetical sequence of these alterations is shown in the diagram (Fig. 21).

of the membranous bags. The latter process would accord better with "autophagy" in organelles carrying out destruction of the now useless empty bags, but it was only rarely seen. The better evidence, in our opinion, points to the process of simple invagination, fractionation and possible solubilization of the empty sacs.

Suggestion of mechanisms for the transformation of insulin from a granular to a more soluble form and for its protected transport to the plasma membrane





Figs. 22–23. Fetal-rat pancreas explanted on the 18th day of gestation, cultured over 4 days and then incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 10 m*M* caffeine and tolbutamide (200 μg/ml). Fig. 22 represents clumps of irregular membranous profiles suggesting a fusion of empty sacs with subsequent formation of autolysosomes, or of a residual body as shown in Fig. 23. $\times$30 000.

must remain even more hypothetical. Little is known about the physico-chemical state of insulin in β-granules, although it might well be constituted of very large insulin polymers. In favour of such an hypothesis one might mention the evidence for association with heavy metals[31] or the ultrastructural evidence of lines of repeating periodicity in the matrix of the granule[32]. Intracytoplasmic solubilization of β-granules might thus

result from a depolymerization process possibly as a consequence of modifications of intracellular pH, as suggested by experiments with isolated β-granules in suspension[33]. Transport through the cytoplasm might result either from simple diffusion following a concentration gradient or be associated with binding to specialized carrier molecules, such as glycoprotein, possibly for protection from the cytoplasmic milieu.

Granular insulin may of course also represent binding of a monomeric or oligomeric form of the hormone with a larger molecule, a concept which might imply an even more complex role of the Golgi apparatus, since, as demonstrated by NEUTRA AND LEBLOND[34] in goblet cells of the intestine, the Golgi complex may be the preferred site of incorporation of carbohydrate molecules into glycoproteins. The role played by carbohydrates in glycoproteins is not clear, but they might facilitate the transport of proteins through the cytoplasm and through the plasma membrane. We therefore suggest that the Golgi complex might fulfill a similar function in the B cell, with binding of insulin to a glycoprotein resulting in granular "packaging" as well as in facilitation of the subsequent exit of the hormone. This hypothesis may recently have received some support from the detection of carbohydrates in a purified fraction of β-granules, as reported by SORENSON *et al.*[35].

What, finally, might be the role of the vesicles with a size intermediate between that of secretory granules and that of microvesicles? Again, we might speculate that these organelles, which are frequently seen after strong stimulation of insulin secretion, are associated with stimulated resynthesis and formed within the Golgi apparatus, possibly containing a more soluble form of insulin. These vesicles thus might represent either non-mature β-granules or a more specific entity which might be involved in direct transfer of newly synthesized insulin through the cytoplasm and in its release.

The largely hypothetical nature of the preceding discussion is clearly understood. Furthermore, we are fully aware of the special character of our preparation which is a *fetal* tissue having been submitted to a *culture* period. The conclusions drawn from our studies are thus not necessarily relevant to other preparations. Nevertheless, our observations do suggest that mechanisms of insulin release other than emiocytosis may well exist and deserve fullest consideration. One such mechanism would be intracytoplasmic dissolution of secretory granules with subsequent—probably protected—transport of insulin through the cytoplasm and the plasma membrane, and also with the subsequent disposal of the empty β-granule membranes.

NOTE ADDED IN PROOF

Since this paper has been submitted for publication, further observations on cultured fetal-rat pancreas incubated for 2 h in a Krebs–Ringer bicarbonate buffer containing 48 m*M* K^+, 5.5 m*M* glucose and 10 m*M* caffeine have provided evidence for a very active emiocytosis in some B cells, therefore suggesting that this process might also account at least for a part of the IRI release from our preparation under strong stimulatory conditions.

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EFFECTS OF VARIOUS *IN VITRO* CONDITIONS ON HEPATIC MICROSOMAL *N*- AND *C*-OXYGENATION OF AROMATIC AMINES*

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SUMMARY

The formation of reactive metabolites from carcinogenic aromatic amines in rat liver is associated with microsomal *N*-oxygenation. *C*-Oxygenation, *i.e.* ring-hydroxylation and *N*-dealkylation, yields only harmless metabolites. In an attempt to elucidate the nature and origin of the reactive metabolites, the mechanism of microsomal *N*- and *C*-oxygenation of aromatic amines has been studied.

Microsomal *C*-oxygenation of dimethylaniline shows a marked substrate optimum followed by a progressive decline; *N*-oxygenation of dimethylaniline and *N*–*C*-transoxygenation of dimethylaniline-*N*-oxide show a continuous rise with increasing substrate concentration. The inhibition of *C*-oxygenation at high substrate concentration is not reversed by washing the microsomes. Large amounts of added *N*-oxygenated metabolite does not affect the *C*-oxygenation. Detergent treatment decreases *C*-oxygenation and *N*–*C*-transoxygenation but increases *N*-oxygenation. Electrophilic agents strongly inhibit *C*-oxygenation but stimulate *N*-oxygenation, leaving *N*–*C*-transoxygenation unaffected.

In the light of these experiments and the electron-donor properties of aromatic amines, the mechanism for *C*- and *N*-oxygenation and the release of electrophilic metabolites associated with the latter process is discussed.

INTRODUCTION

The formation of reactive metabolites of carcinogens, and their interference

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Abbreviations: ADP, adenosine diphosphate; DMA, *N,N*-dimethylaniline; DMA-*N*-oxide, *N,N*-dimethylaniline-*N*-oxide; FAD, flavine adenine dinucleotide; G-6-P, glucose 6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; MeHgOH, methylmercurihydroxide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PCA, perchloric acid; TCA, trichloroacetic acid.

with the functions of information-carrying macromolecules in target cells, is considered to be of basic importance in chemical carcinogenesis. During the conversion of hepato-carcinogens such as aromatic amines to excretable conjugates in the endoplasmic reticulum of liver cells[1], electrophilic intermediates may be formed, which bind to proteins and nucleic acids[2,3], primarily in this organelle[4-6]. These reactions lead at an early stage to structural disorganization[7-10] and functional disturbances[9,11-16] of the endoplasmic reticulum, as well as of the ribosomes attached to this structure.

The chemical nature of these reactive derivatives is by no means clarified. Microsomal oxygenation of aromatic amines in the presence of suitable electron donors can take place either on the nitrogen atom (*N*-oxygenation)[17-23] or on different carbon atoms yielding ring-hydroxylated or *N*-dealkylated products (*C*-oxygenation)[24,25]. The former reaction is mediated through a flavoprotein enzyme, whereas the latter[23,26] in addition to this enzyme is also dependent on cytochrome P450. As *N*-oxygenated derivatives are metabolized *in vivo* and *in vitro* to the same ring-hydroxylated and *N*-dealkylated products as the parent amines themselves[18,20,27-32], it has been postulated by some authors[20,22,29] that the *N*-oxygenated metabolites are intermediates in the NADPH-dependent *C*-oxygenation of aromatic amines. Experiments contradictory to this view have, however, been presented[25,33].

Current evidence suggests that *N*-oxygenated derivatives of carcinogenic amines are proximal carcinogenic metabolites. They have a high carcinogenic potency[34,35], and a greater ability than the parent amines and *C*-oxygenated metabolites to react with nucleic acids and proteins *in vivo*[36,37]. Moreover the urinary output of *N*-oxygenated metabolites increases during the continuous feeding of carcinogenic amines[38]. This effect is accompanied by a shift in the microsomal detoxication pattern of aromatic amines *in vitro* from *C*- to *N*-oxygenation[11]. A proportional increase in microsomal *N*-oxygenation was also associated with those environmental, inherent and endocrine conditions which increase the carcinogenic potency of the amines[11,39-43], and the output of urinary *N*-oxygenated metabolites[38,44-46]. Concomitantly the effects of carcinogenic amines on microsomal protein synthesis were accentuated[13-15,47]. However, the *N*-oxygenated derivatives themselves lack ability to bind to proteins or nucleic acids[48,49] at physiological pH in model systems, and do not affect microsomal protein-synthesizing activities when added *in vitro*[50]. An intermediate in the *N*-oxygenation[11] or a product of further conversion of the *N*-oxygenated derivatives[18,50] have therefore been postulated as ultimately responsible for the tumour-inducing action of carcinogenic amines.

In the present investigation the general mechanism for microsomal *C*- and *N*-oxygenation of aromatic amines has been studied in order to clarify the nature and origin of the reactive metabolites of the carcinogenic members of this group. As in the previous study on *in vivo* effects[11], the monocyclic noncarcinogenic amine DMA was used as a test substrate thus avoiding the damage to microsomal detoxication mechanisms demonstrated with low concentrations of carcinogenic amines *in vitro*[51] (*cf.* ref. 18). The results support the view that in *C*-oxygenation P450 is activated

to form a ternary complex[52] with oxygen and substrate by a flavoprotein-catalyzed electron transfer from the amine substrate. The *C*-oxygenated product is released from P450 without the formation of an intermediary *N*-oxygenated metabolite by a flavoprotein-mediated electron transfer from NADPH. When the transfer of electrons from flavoprotein to P450 is inhibited, oxygen enters as a relatively less efficient electron acceptor at the flavoprotein level. Under these conditions, the substrate is oxygenated on the nitrogen atom. During this reaction electrophilic metabolites may be formed which react with nucleophilic centers in adjacent structures.

Preliminary presentations of these data have been published previously[53–55].

MATERIALS AND METHODS

Animals

Male rats (150–200 g) of a Sprague Dawley strain were purchased from Anticimex AB, Rotebro (Sweden) and fed Anticimex rat pellets No. 210; they were starved for 16–20 h before being killed.

Chemicals

ADP, G-6-P, G-6-PDH, NADP, *N*-ethylmaleimide and *p*-chloromercuriphenylsulphonic acid were obtained from Sigma Chemical Company, St. Louis, Mo; DMA-*N*-oxide from K & K Laboratories, Plainview, N.Y.; Ficoll from Pharmacia AB, Uppsala (Sweden); MeHgOH and phenylmercuridicyandiamide from Casco AB, Stockholm (Sweden).

Preparation and pretreatment of microsomes

Microsomes were prepared from rat livers, as described in a previous paper[11]. The homogenization medium was 0.2 *M* potassium phosphate buffer pH 7.5 unless otherwise mentioned. The microsomes were washed by resuspension to the original volume in medium and recentrifugation for 55 min at 105 000 *g*.

Pretreatment of microsomes with cholate was for 1 h at 0°.

For ultrasonication 3-ml batches of microsomal suspensions in ice-chilled test tubes were treated for 2-min periods in an MSE ultrasonic disintegrator (20 kcycles/sec, 60 W). Samples of the pooled batches were taken at different periods; the remaining suspension was again divided in 3-ml batches before continuing sonication.

In the preincubation experiments the recentrifugation was omitted. After preincubation in the above phosphate medium or in citrate medium (0.1 *M* sodium citrate in 0.1 *M* potassium phosphate buffer pH 7.5), 6 ml of the incubation mixture was layered over 5 ml of 5 % Ficoll in 0.2 *M* potassium phosphate buffer pH 7.5 and centrifuged for 55 min at 105000 *g*. The pellet of washed microsomes was resuspended in the citrate medium and used in the final incubation.

Oxidative N-demethylation and N-oxide formation

When not otherwise stated, the incubation system contained in final volumes of 2 ml: 0.2 ml microsomal suspension (1.5–3 mg microsomal protein), 0.1 μmoles NADP, 10 μmoles G-6-P, 120 μmoles nicotinamide, 0.25 Kornberg Units G-6-PDH,

10 μmoles DMA and 200 μmoles/ml of potassium phosphate buffer pH 7.5.

The cofactors were premixed before addition to the incubation; with this low concentration of NADP all the coenzyme was kept reduced during the incubation. The nicotinamide added to enable the use of low NADP concentration caused only a minor decrease in enzyme activity as compared with a system with 10-fold NADP concentration in absence of nicotinamide. The incubations were carried out aerobically with gentle shaking at 35°. The reaction was terminated by the addition of 1 ml 0.9 *M* PCA. In some early experiments on oxidative demethylation the incubations were terminated by the addition of 1 ml 20 % TCA. This difference in procedure did not affect the final analytical result.

N-Demethylation of DMA-N-oxide

When not otherwise mentioned the incubations contained in a final volume of 2 ml: 0.2 ml microsome suspension (2–3 mg protein), 20 μmoles DMA-*N*-oxide and 200 μmoles/ml of potassium phosphate buffer pH 7.5. Other conditions were the same as for *N*-demethylation of DMA.

Enzymatic NADPH-dependent lipid peroxidation

The conditions were similar to *N*-demethylation except that DMA was omitted from the incubation, and that non-washed microsomes were used.

N-Demethylation and *N*-oxygenation of DMA, *N*-demethylation of DMA-*N*-oxide and lipid peroxidation showed a linear dependence on the concentration of microsomes within the limits used in the experiments.

Analytical methods

All analytical methods were as described in a previous report[11].

RESULTS

Effects of various incubation media on the oxidative N-demethylation and N-oxygenation of DMA

Fig. 1 illustrates the time course of (*a*) oxidative *N*-demethylation, (*b*) *N*-oxygenation, and (*c*) total oxygenation of DMA in incubation systems containing either 0.2 *M* potassium phosphate buffer or 0.175 *M* KCl in 0.035 *M* Tris buffer pH 7.5 (35°). In phosphate medium these functions proceed almost linearly over a period of 60 min. In Tris–KCl medium the period of linearity was much shorter and already after 10 min there was no further increase in the total amount of oxygenated amine (Fig. 1c). When the incubations in Tris–KCl medium were fortified with ADP, the linearity of the reactions was markedly extended, although the efficiency of the oxygenation reactions was smaller than in the phosphate medium. In potassium phosphate medium ADP had no effect. When the microsomes were incubated in a medium of 0.25 *M* sucrose in 0.035 *M* Tris buffer pH 7.5 (35°) the results were similar to those obtained using Tris–KCl medium[56].

In the presence of ADP increasing concentration of phosphate did not influence

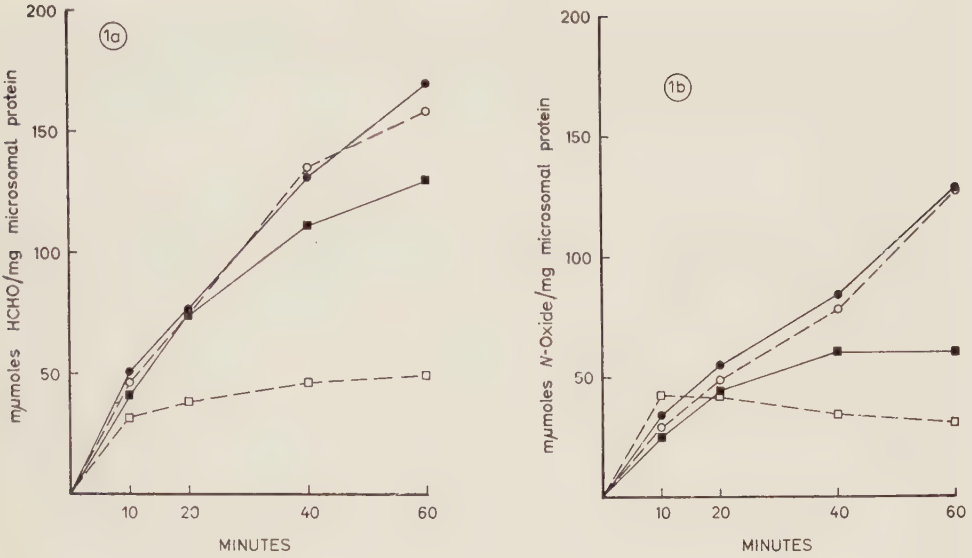


Fig. 1. Influence of incubation medium on oxidative *N*-demethylation (*a*), N-oxygenation (*b*) and total oxygenation (*c*) of DMA by rat-liver microsomes. Washed microsomes were prepared in 0.2 *M* potassium phosphate buffer pH 7.5 (○, ●) or 0.175 *M* KCl in 0.035 *M* Tris buffer p H7.5 (35°) (□, ■) and incubated in the same final concentration of the respective medium. ●, ■, ADP (1.0 μmoles/2 ml) added to the incubation mixture. All incubations were at 35° and contained in a final volume of 2 ml: 0.1 μmoles NADP; 10 μmoles G-6-P; 0.25 Kornberg Units G-6-PDH; 120 μmoles nicotinamide; 10 μmoles DMA and 2.4 (phosphate buffer) or 2.9 (Tris–KCl buffer) mg microsomal protein.

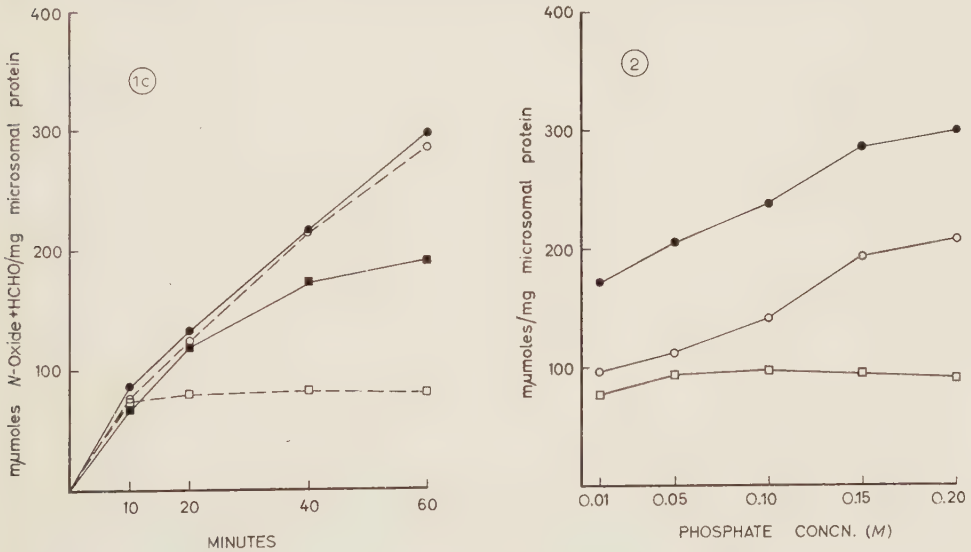


Fig. 2. Influence of phosphate concentration on *N*-demethylation (mμmoles HCHO/mg microsoma protein; ○), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein; □), and total oxygenation (mμmoles HCHO + DMA-*N*-oxide/mg microsomal protein; ●), of DMA by rat-liver microsomes. Washed microsomes were prepared in 0.1 *M* potassium phosphate buffer pH 7.5. Final concentration of potassium phosphate buffer in the incubations as indicated. All other incubation conditions as in Fig. 1, except that ADP (1.0 μmoles/2 ml) and Tris buffer pH 7.5 (40 μmoles/2 ml) were added to all incubations. Microsomal concentrations: 3.3 mg protein/2 ml. Incubation period 40 min.

the *N*-oxygenation of DMA, while the *N*-demethylation was appreciably stimulated (Fig. 2). As a result the *N*-oxygenation/*C*-oxygenation quotient is somewhat decreased. In view of these data a phosphate concentration of 0.2 *M* was used in the further experiments unless otherwise indicated.

Effect of substrate concentration

Microsomal systems were incubated with increasing amount of substrate and their capacity to perform *N*-oxygenation and oxidative *N*-demethylation of DMA and *N*-demethylation of DMA-*N*-oxide was measured (Fig. 3). The *N*-oxygenation of DMA as well as the *N*-demethylation of DMA-*N*-oxide increased continuously with increasing substrate concentration up to 50 μmoles/ml or more at the microsomal concentration used. The oxidative *N*-demethylation of DMA showed a more unusual response to increased substrate levels. Up to a DMA concentration of 5 μmoles/ml the demethylation increased more rapidly than the *N*-oxygenation; this concentration is, however, optimal for *C*-oxygenation. Higher substrate concentrations caused a sharp decline in the reaction velocity, and at 50 μmoles/ml the activity was only 50–70 % of the optimum value. As a result the *N*-oxygenation/*C*-oxygenation quotient exhibited a minimum at 5 μmoles DMA per ml followed by a rapid rise. The total oxygenation of DMA increased up to a DMA concentration of 5 μmoles/ml.

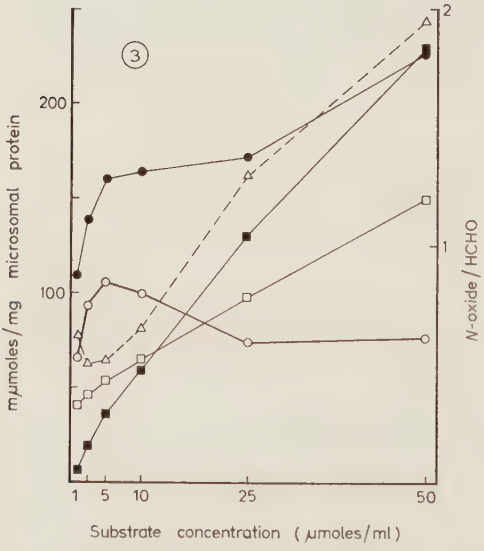


Fig. 3. Effect of substrate concentrations on *N*-demethylation (mμmoles HCHO/mg microsomal protein; ○), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein; □), total oxygenation (mμmoles HCHO + DMA-*N*-oxide/mg microsomal protein; ●) and *N*-oxygenation/*N*-demethylation ratio (△) of DMA, and *N*-demethylation of DMA-*N*-oxide (mμmoles HCHO/mg microsomal protein; ■) by rat-liver microsomes. Washed microsomes were prepared in 0.2 *M* potassium phosphate buffer pH 7.5. All incubations were at 35° for 40 min, and contained in a final volume of 2 ml: (*a*) Substrate DMA: 0.1 μmoles NADP; 10 μmoles G-6-P; 0.25 Kornberg Units G-6-PDH, 120 μmoles nicotinamide, 2.13 mg microsomal protein, DMA as indicated and 200 μmoles/ml potassium phosphate buffer pH 7.5. (*b*) Substrate DMA-*N*-oxide: 2.13 mg microsomal protein; DMA-*N*-oxide as indicated and 200 μmoles/ml potassium phosphate buffer pH 7.5.

At intermediate concentrations the curve tended to form a plateau, but then again increased, indicating that the enhanced *N*-oxygenation overcompensated the decrease in *C*-oxygenation.

Influence of pretreatment of microsomes with cholate or ultrasonication

ZIEGLER AND PETTIT[22] have shown that rat-liver microsomes which have been subject to "ageing" or cholate treatment show an increased capacity to form *N*-oxide from DMA, whereas their capacity to oxidatively *N*-demethylate this substrate is strongly decreased. Fig. 4a shows the *N*-oxygenation, oxidative demethylation and total oxygenation of DMA by microsomes pretreated with different concentrations of cholate. Whereas the *N*-demethylation showed a strong progressive decline above a cholate concentration of 0.05 mg/mg microsomal protein*, the activity of *N*-oxygenation increased to a peak at 0.25 mg cholate/mg microsomal protein. At higher concentrations the activity was again reduced, although a concentration as high as 3–4 mg cholate per mg microsomal protein was needed in order to reduce the activity to the level of untreated microsomes. The initial rise in *N*-oxygenation was greater than the inhibition of the *C*-oxygenation; thus the total oxygenation was doubled at cholate

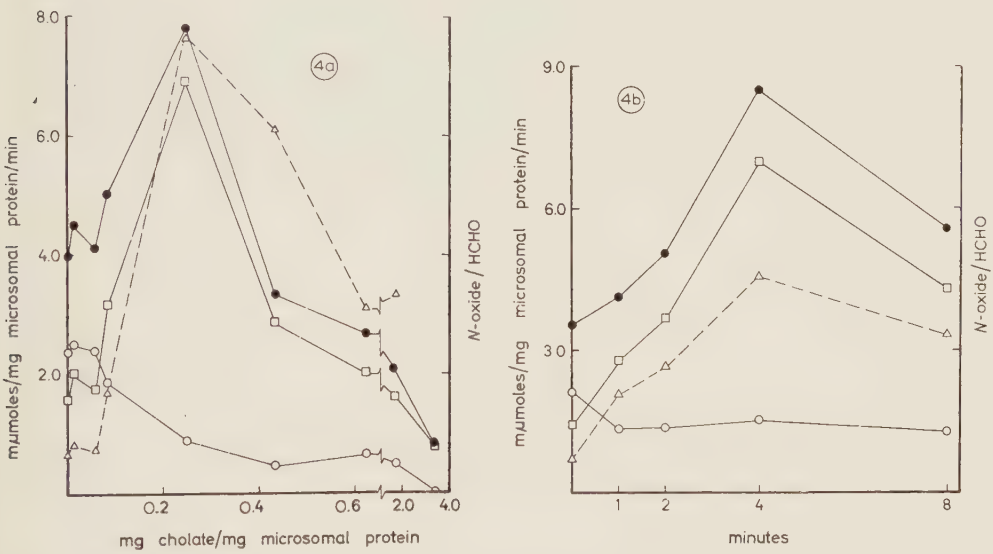


Fig. 4. Effect of pretreatment of microsomes with sodium cholate (*a*) or with ultrasonic vibrations (*b*) on *N*-demethylation (mμmoles HCHO/mg microsomal protein/min; ○), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein/min; □), total oxygenation (mμmoles HCHO + DMA-*N*-oxide/mg microsomal protein/min; ●), and *N*-oxygenation/*N*-demethylation ratio (△) or DMA by rat-liver microsomes. Incubation conditions as in Fig. 3 with 10 μmoles DMA/2 ml as substrate and 2.4 (cholate) or 2.6 (ultrasonication) mg microsomal protein/ml. Cholate pretreatment for 1 h at 0° at the cholate concentrations indicated. Ultrasonication (20 kcycles/sec) for the time periods indicated.

* The slight increase in activity of *C*-oxygenation of DMA and DMA-*N*-oxide as well as *N*-oxygenation of DMA obtained with low cholate concentration is reproducible; it will, however, not be discussed here.

concentrations optimal for *N*-oxygenation. At a slightly higher cholate concentration, however, the total oxygenation decreased below that of the control. The *N*-oxygenation/*C*-oxygenation quotient was increased by a factor of 10 at the *N*-oxygenation optimum; although decreased at higher cholate concentrations, it always remained well above the control level.

Fig. 4b shows the same oxygenation reactions with microsomes pretreated for various periods with ultrasonication. The effects, although less marked, were similar to those obtained with cholate treatment; the activity of *N*-demethylation decreased progressively with increasing time of ultrasonication, while the *N*-oxygenation activity showed a maximum in microsomes treated for about 4 min. Thereby the possibility of a substrate effect of cholate is excluded. This possibility is also negated by the slight activity increase at low cholate concentrations mentioned above.

A comparison between the *N*-demethylation of DMA and DMA-*N*-oxide after cholate pretreatment is shown in Fig. 5. The demethylation of the *N*-oxide showed a similar although somewhat less marked decline than the demethylation of the parent amine.

Effects of organic mercurials and N-ethylmaleimide

Fig. 6a shows the effect of MeHgOH on microsomal *N*-oxygenation and *N*-demethylation. At 0.03 m*M* the mercurial had no effect on either *N*- or *C*-oxygenation of DMA. At 0.1 m*M*, however, the oxygenation pattern of DMA showed a striking change; whereas the *C*-oxygenation dropped to 50 % of the control value, the *N*-oxygenation was more than doubled. At higher mercurial concentrations both activities decreased. However, concentration of 1.0 m*M* was needed to bring the *N*-oxygen-

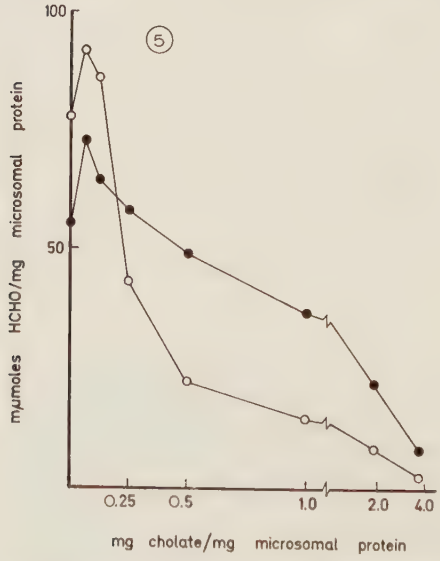


Fig. 5. Effect of sodium cholate pretreatment on *N*-demethylation of DMA (○) or DMA-*N*-oxide (●) by rat-liver microsomes. Incubation conditions as in Fig. 3 with 10 μmoles DMA or 20 μmoles DMA-*N*-oxide per 2 ml as substrate. Microsomal concentration: 2.4 mg protein per 2 ml. Cholate treatment as in Fig. 4.

ation down to the control level. At this concentration *C*-oxygenation was already totally inhibited. At lower MeHgOH concentrations the rise in *N*-oxygenation was greater than the decrease in *C*-oxygenation activity; thus the total oxygenation first increased and then dropped off sharply at higher MeHgOH concentrations. As a result of this preferential effect of the mercurial on the *C*-oxygenation the *N*-oxygenation/*C*-oxygenation quotient increased progressively and at 0.3 m*M* MeHgOH reached a value 20 times higher than the control; above this mercurial concentration it cannot be calculated because of the total absence of *C*-oxygenation.

The *N*-demethylation of DMA-*N*-oxide was influenced by the added mercurial only at concentrations above 1.0 m*M* which totally abolished the demethylation of DMA, but only slightly reduced the demethylation of the *N*-oxide.

Similar effects on the oxygenation of DMA were obtained with the mercurials *p*-chloromercuriphenylsulphonic acid and phenylmercuridicyandiamide. With *N*-ethylmaleimide the effects were less marked and were obtained only at 10 times higher concentrations (Fig. 6b).

Persistence of N- and C-oxygenation at different substrate levels

The time course of the microsomal *N*- and *C*-oxygenation of DMA was determined at substrate concentrations optimal (5 μmoles DMA/ml) or inhibitory (25 μmoles DMA/ml) for *C*-oxygenation (Fig. 7). The *N*-oxygenation proceeded linearly at both substrate levels for more than 60 min. The same holds true for the *N*-demethylation at the lower DMA concentration, but with 25 μmoles/ml of DMA, the *N*-deme-

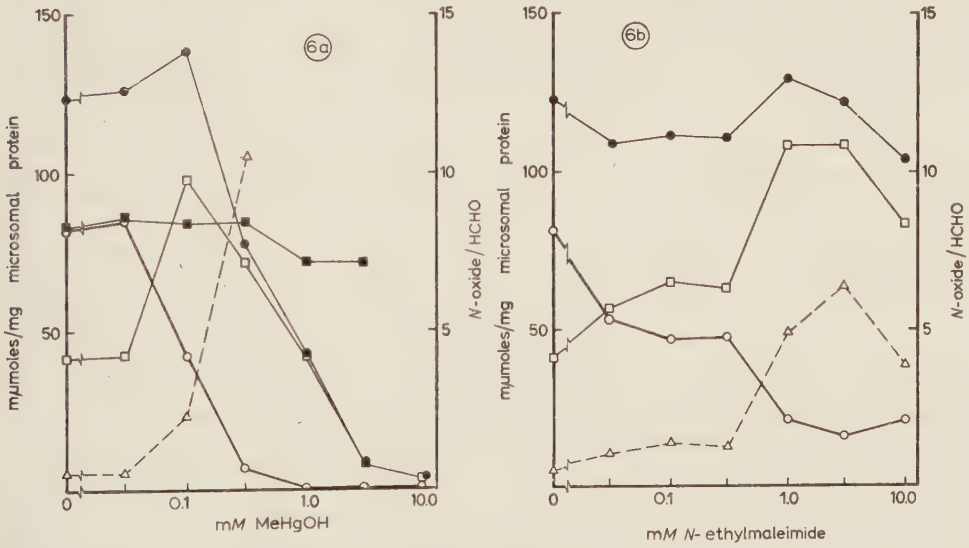
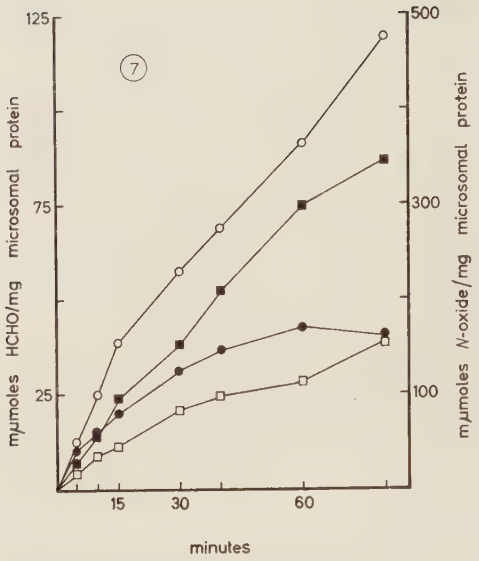


Fig. 6. Effect of MeHgOH (*a*) or *N*-ethylmaleimide (*b*) on *N*-demethylation (mμmoles HCHO/mg microsomal protein; ○), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein; □), total oxygenation (mμmoles HCHO + DMA-*N*-oxide/mg microsomal protein; ●), and *N*-oxygenation/*N*-demethylation ratio (△) of DMA and *N*-demethylation of DMA-*N*-oxide (mμmoles HCHO/mg microsomal protein; ■), by rat-liver microsomes. Incubation conditions as in Fig. 3 with 10 μmoles DMA or 20 μmoles DMA-*N*-oxide per 2 ml as substrate. Microsomal concentration: 2.7 mg protein per 2 ml. MeHgOH or *N*-ethylmaleimide as indicated.



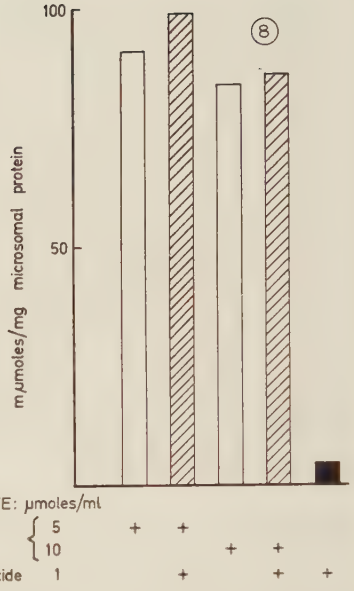


Fig. 7. *N*-Demethylation (○, ●) and *N*-oxygenation (□, ■) of DMA by rat-liver microsomes, as a function of incubation period. Incubation conditions as in Fig. 3 with 10 μmoles (○, □) or 50 μmoles (●, ■) DMA per 2 ml as substrate. Incubation periods as indicated. Microsomal concentration: 2.9 mg protein per 2 ml.

Fig. 8. Effect of added DMA-*N*-oxide on *N*-demethylation of DMA (mμmoles HCHO/mg microsomal protein) by rat-liver microsomes. Incubation conditions as in Fig. 3 with 10 or 20 μmoles DMA per 2 ml added as substrate. Addition of DMA-*N*-oxide (2 μmoles per 2 ml) as indicated. Microsomal concentrations: 2.6 mg protein per 2 ml.

thylating activity began to decline within 5-min incubation; after 60 min it had completely vanished.

Effect of added DMA-N-oxide on the N-demethylation of DMA

DMA-*N*-oxide in 5–10 times higher concentrations than that obtained after 40-min incubation with 25 μmoles/ml of DMA was included in the incubation system. This addition did not inhibit the *N*-demethylation of DMA under otherwise optimal conditions (Fig. 8). The small increment in formaldehyde production observed is accounted for by the *N*-demethylation of the DMA-*N*-oxide itself.

Effect of preincubation of microsomes with varied concentrations of DMA

In order to test whether the inhibitory effect of high DMA concentrations on the microsomal *N*-demethylation of DMA was reversible, microsomes were preincubated with optimal and inhibitory concentrations of DMA in the presence of a NADPH-generating system. The microsomes were washed by centrifugation through a Ficoll gradient and their *N*-oxygenation and *C*-oxygenation activity were tested in a second incubation with optimal DMA concentration. Since lipid peroxidation which

may occur in microsomal systems at low substrate concentrations may disturb the functions of the membrane-bound enzyme system, it was considered essential to keep this factor under control. Fig. 9 shows that the presence of 0.1 *M* citrate in the preincubation medium caused a 10-fold decrease in lipid peroxidation in the absence of DMA. The presence of DMA (5 μmoles/ml) alone inhibited lipid peroxidation (*cf.* ref. 11), and a further decrease was obtained in the presence of citrate. This effect was correlated with an increased stability of the microsomes, manifesting itself in the second incubation as a decreased *N*-oxygenation/*C*-oxygenation quotient, and a small increase in the total oxygenation. When no DMA was present during preincubation this stabilization was accounted for by an increased activity of both *N*- and *C*-oxygenation, the latter being more marked. In presence of DMA only *C*-oxygenation was enhanced. Even when DMA and citrate were present in the preincubation, the *N*-oxygenation/*C*-oxygenation quotient was higher with preincubated

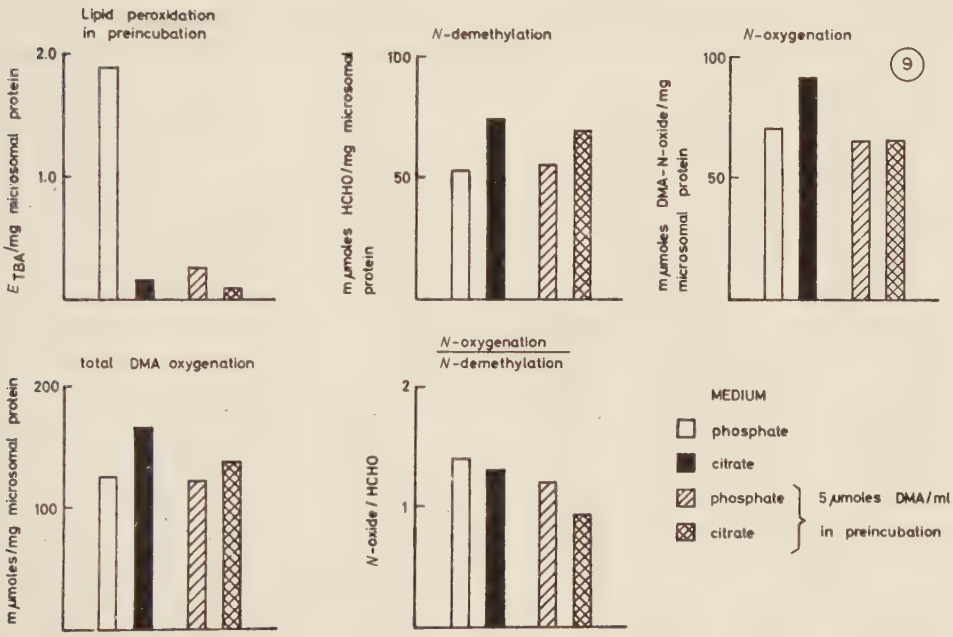


Fig. 9. Effect of preincubation of rat-liver microsomes in phosphate (0.2 *M* potassium phosphate buffer pH 7.5) or citrate (0.1 *M* sodium citrate, 0.1 *M* potassium phosphate buffer pH 7.5) medium in absence or presence of DMA on their activity in *N*-demethylation, *N*-oxygenation, total oxygenation and *N*-oxygenation/*N*-demethylation ratio of DMA determined in a second incubation after washing. Lipid peroxidation during preincubation shown in first graph. Microsomes were prepared in 0.2 *M* potassium phosphate buffer pH 7.5. Preincubations were for 30 min at 35° and contained in a final volume of 8 ml: 0.4 μmoles NADP; 40 μmoles G-6-P; 1.0 Kornberg Units G-6-PDH; 480 μmoles nicotinamide; 10.8 mg microsomal protein, DMA as indicated and 200 μmoles/ml potassium phosphate buffer pH 7.5 (phosphate medium) or 100 μmoles/ml potassium phosphate buffer pH 7.5 and 100 μmoles/ml sodium citrate pH 7.5 (citrate medium). Final incubations (incubation volume 2 ml) were under the same conditions as preincubation in citrate medium with 10 μmoles DMA/2 ml as substrate and 1.4 mg microsomal protein obtained from the preincubation mixture by centrifugation through 5% Ficoll in 0.2 *M* potassium phosphate buffer pH 7.5.

than with untreated microsomes. As will be shown in a subsequent report[56] this increase was caused by the washing procedure rather than the preincubation *per se*.

The inhibitory effect of high DMA concentrations on *C*-oxygenation was not reversed by the washing procedure (Fig. 10). The *N*-demethylation during the second incubation was reduced by more than 50 % when the DMA concentration in the preincubation had been increased from 5 to 25 μmoles/ml. Although the rise in *N*-oxygenation was not as marked as in a single direct incubation, the *N*-oxygenation/*C*-oxygenation quotient was more than doubled.

Effect of NADPH on the N-demethylation of DMA-N-oxide

In contrast to oxidative *N*-demethylation of DMA the *N*-demethylation of DMA-*N*-oxide is independent of an extraneous electron donor[29,31]. Fig. 11 shows the substrate dependence of this reaction in the presence and absence of a NADPH-generating system. As was shown already in Fig. 3 the demethylation of DMA-*N*-oxide in the absence of electron donor increased linearly with increasing substrate concentration. In the presence of NADPH, however, the activity gradually leveled off at concentrations above 2 μmoles DMA-*N*-oxide/ml, and at the microsomal

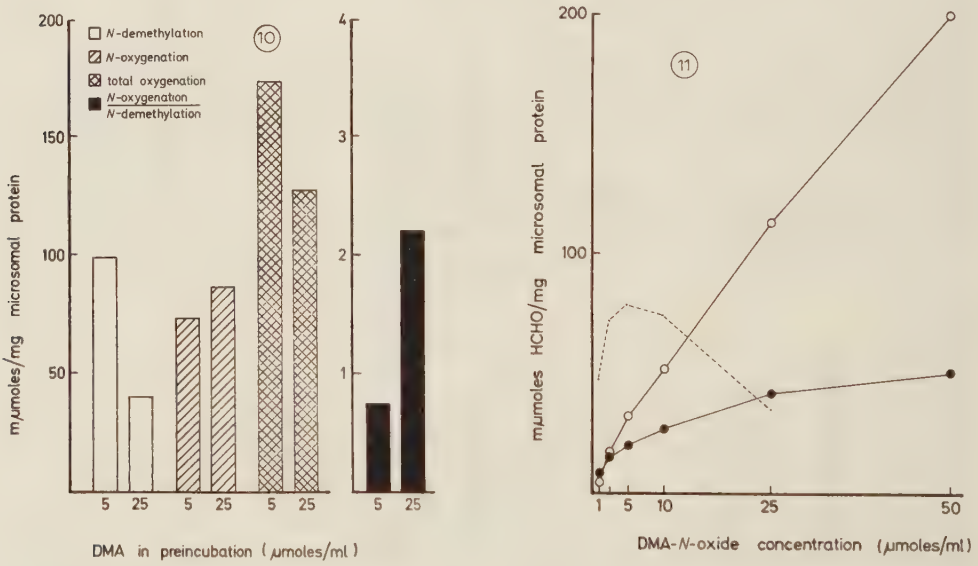


Fig. 10. Effect of preincubation of rat-liver microsomes with various amounts of DMA on their activity in *N*-demethylation (mμmoles HCHO/mg microsomal protein), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein), total oxygenation (mμmoles HCHO + DMA-*N*-oxide/mg microsomal protein) and *N*-oxygenation/*N*-demethylation ratio of DMA during a second incubation after washing. Preincubation conditions as in Fig. 9 with citrate medium, DMA concentration as indicated and 11.6 mg microsomal protein/8 ml. Final incubation as in Fig. 9 with 1.4–1.6 mg microsomal protein/2 ml.

Fig. 11. *N*-Demethylation of DMA-*N*-oxide by rat-liver microsomes in presence (●) or absence (○) of a NADPH-generating system as a function of substrate concentration. Incubation conditions as in Fig. 3*a*, except with DMA-*N*-oxide instead of DMA (●) or as in Fig. 3*b* (○) ·————·, *N*-demethylation of DMA included as comparison (incubation conditions as in Fig. 3*a*). Microsomal concentration, 3.1 mg protein/2 ml.

concentration used showed a tendency to plateau formation at substrate concentrations higher than 25 μmoles/ml.

As compared with the oxidative *N*-demethylation of DMA at optimal substrate concentration (5 μmoles/ml) the demethylation of DMA-*N*-oxide in these experiments manifested itself as a relatively slow process. At a substrate concentration of 5 μmoles/ml the activity in the absence of NADPH was only about 40 % of the DMA demethylation activity in a complete system and in the presence of NADPH only 25 %. With increasing concentrations of DMA-*N*-oxide the demethylation of this substrate in the absence of NADPH fairly soon surpassed the optimal DMA demethylations. However, in the presence of the electron donor (*i.e.* incubation conditions identical with those of DMA-demethylating systems), this never occurred.

DISCUSSION

The optimum conditions for microsomal oxygenation reactions, *e.g.* *C*-oxygenation and *N*-oxygenation of aromatic amines and NADPH-dependent lipid peroxidation, have been studied by several investigators. However, little attention has been directed on the effects of variations in medium composition (*cf.* ref. 57), and thus KCl, sucrose or phosphate media have in most cases been used indiscriminately. The lack of linearity for these reactions in Tris–KCl or Tris–sucrose and the stimulating effect of ADP (Fig. 1)[56] are not obtained when L-gulonolactone is substituted for NADPH as electron donor[58]. Experiments to be published in a forthcoming report[56] indicate that the lack of linearity of these reactions in Tris–KCl and Tris–sucrose media is due to a Mg^{2+}-dependent microsomal pyrophosphatase (*cf.* ref. 59), cleaving NADPH into its two constituent nucleotides. The reduced nicotinamide nucleotide is afterwards dephosphorylated to reduced nicotinamide riboside. The breakdown of NADPH is inhibited by chelating agents like ADP or large concentrations of phosphate. These effects should be kept in mind when comparing different results on microsomal oxygenation reactions where NADPH has been used as electron donor. The mechanism of enhancement by increased phosphate concentration of *C*-oxygenation in the presence of ADP (Fig. 2) is not yet clear. The selectivity of the effect indicates an activation of some functional component in the *C*-oxygenation pathway which is not common to *N*-oxygenation. A similar effect of phosphate has been observed by SATO *et al.*[60] on the steapsin-solubilized flavoprotein exhibiting a naphthoquinone-stimulated NADPH-oxidase activity, which they consider to be a constituent of the *C*-oxygenation enzyme chain.

ZIEGLER *et al.*[22,29] as well as UEHLEKE[20] have proposed that the *N*-oxygenated metabolites of aromatic amines are intermediates in the *C*-oxygenation of these substances; by a Bamberger rearrangement[61] the *N*-linked oxygen would be transferred to a carbon. According to this view the oxygenating enzyme should be the flavoprotein, whereas the P450 enzyme should act as an intramolecular oxygen transferase. This interpretation is supported by their finding that in intact microsomes the demethylation of the proposed intermediate DMA-*N*-oxide is more rapid than the demethylation of DMA itself. The present experiments (Fig. 11) indicate, however,

that this is true only in the absence of electron donor, and when DMA-*N*-oxide is added at 2–5 times higher concentrations than the substrate optimum for DMA demethylation. When both reactions are compared in the presence of NADPH and at substrate concentrations where both reactions show linearity, DMA is demethylated 4 times more rapidly than DMA-*N*-oxide. Even if the concentration of the DMA-*N*-oxide is increased by a factor of 10, the demethylation of this substrate is only about 60 % as active as optimal DMA demethylation. This is in keeping with BICKEL's experiments[62] which indicate that iprimamine-*N*-oxide in contrast to iprimamine, is not demethylated by rat-liver microsomes.

It is shown in Fig. 6 that *C*-oxygenation of DMA is totally abolished by MeHgOH under conditions when neither of the two postulated partial reactions (*i.e.* *N*-oxygenation of DMA and demethylation of DMA-*N*-oxide) were markedly affected. In this context KAMPFFMEYER AND KIESE[33] reported a 40 times higher K_m value for oxygen in the *N*-oxygenation of aniline than that in the *C*-oxygenation of the same substrate. Furthermore MCMAHON AND SULLIVAN[63] using labeled amines in experiments on microsomal demethylation observed no accumulation of radioactivity in an added pool of cold amine-*N*-oxide. These experiments support the concept that *N*-oxygenation in combination with a subsequent *N*-demethylation of the *N*-oxygenated metabolite represents only a rather inefficient alternative pathway for *C*-oxygenation of aromatic amines.

The parallel decreases obtained with cholate pretreatment in the ability of microsomes to demethylate DMA and DMA-*N*-oxide (Fig. 5) suggests that both of these reactions are dependent on a functional coupling between the flavoprotein and cytochrome P450. The decrease in intact P450 after treatment with deoxycholate[29] seems insufficient to account for the inhibition of demethylation activity; even after prolonged pretreatment of the microsomes under conditions which almost completely abolish the activity of *N*-oxide demethylation, the P450 level is still higher than 30 % of the control value[29].

The inhibitory effect of high substrate concentrations on the microsomal *C*-oxygenation of DMA (Fig. 3) became stronger with longer incubation (Fig. 7). This fact suggests that the effect was due to the production of an inhibitory metabolite during incubation. The experiments shown in Figs. 7 and 8 indicate, however, that this metabolite was not identical with the *N*-oxide. The inhibition was manifest already after 10 min of incubation with 25 μmoles DMA/ml, when the concentration of *N*-oxide was still comparatively small (Fig. 7, *cf.* Fig. 3). Much higher concentrations of DMA-*N*-oxide failed to inhibit the *C*-oxygenation of DMA, when added at the beginning of the incubation (Fig. 8). Since the activity of the inhibited microsomes was not restored after washing (Fig. 10) the inhibition was obviously not due to any metabolite accumulating in the medium, but rather to irreversible damage to a component in the chain of microsomal enzymes by some reactive intermediate formed during the *N*-oxygenation reaction.

The *C*-oxygenation was much more vulnerable to electrophilic agents (organic mercurials or *N*-ethylmaleimide) than the *N*-oxygenation (Fig. 6, *cf.* ref. 64). The similarity between the inhibitory pattern of these agents and that of high substrate

concentrations points to an electrophilic intermediate in the *N*-oxygenation as the actual inhibitor of the *C*-oxygenation reaction. This is in agreement with the electron-donor properties of aromatic amines[65]. EVANS[66] demonstrated that this property may cause the formation of a labile adduct between DMA and molecular oxygen. MULLIKEN and his group[67] showed that, because of the relatively poor electron-acceptor capacity of O_2 in this system, this reaction involves a "less than one electron" transfer, completely reversed at reduced oxygen tension. Using the more efficient electron acceptors diacyl peroxides, HORNER[68] was able to demonstrate that complexes involving ammonium radical cations are formed under analogous conditions. By the release of a labilized α-hydrogen atom and subsequent filling up of the electron doublet of the nitrogen atom, these assume the character of carbon radicals. In the microsomal enzyme chain an efficient electron acceptor is provided by the oxidized flavoprotein which by accepting one electron is converted to a semiquinone or free radical[69,70]. Thus *N*-oxygenation as well as *C*-oxygenation, which both involve flavoprotein electron transfer[22,23], could also imply the formation of similar complexes of substrate and flavoenzyme[71]. A mechanism has been inferred[72,73] where *C*-oxygenation involves a further electron transfer from flavin to P450, paralleled by a transfer of an adduced ammonium cation radical; P450 is then activated to adduce oxygen with the formation of a ternary complex[52]. According to HORNER[68,74] ammonium radical cations by loss of a labilized proton and an electron redistribution assume the character of carbon radicals with a center of low electron density in α-*ortho*- or *para*-position. It has further been inferred[72,73] that the *C*-oxygenated metabolite is released from the enzyme by a further flavin-mediated electron transfer from NADPH. The structure of the amine determines whether the unpaired electron and thereby the hydroxylation will appear in α-*ortho*- or *para*-position[68,74].

The *N*-oxygenation of aromatic amines[23,26] can proceed in the absence of functional P450. Thus the flavoprotein alone is responsible for the oxygen activation in this reaction (*cf.* ref. 70). The observation of KAMPFFMEYER AND KIESE[33] that the K_m value for oxygen is severalfold larger in *N*-oxygenation than in *C*-oxygenation of the same amine suggests that the increased *N*-oxygenation under conditions of inhibited electron transfer from flavoprotein to P450 (Figs. 4, 6) may be the outcome of a decreased competition for electrons between oxygen and P450.

SCRIBNER *et al.*[75] and HIGASHINAKAGAWA *et al.*[76] observed that the binding of reactive metabolites of carcinogenic amines to methionine sulfur occurs at the same positions (*i.e.* α and *ortho*) on the amine molecule as those where unpaired electrons appear in the formation of carbon radicals[68,74]. This supports the idea[72,73] that the electrophilic metabolites formed in association with *N*-oxygenation are such radicals. The parallel between effects of electrophilic agents, *i.e.* MeHgOH or *N*-ethylmaleimide, and elevated substrate levels on *C*-oxygenation infers that the inhibition of *C*-oxygenation in the latter case is due to an interaction of these electrophilic metabolites with nucleophilic centers in the enzyme structures[72,73].

The attacked nucleophilic center in the *C*-oxygenation enzyme chain may be a sulphydryl group, but this cannot be conclusively stated on the basis of the present

experiments since organic mercurials are not strictly specific for these groups[77].

The need for functional intactness of the flavoprotein–P450 enzyme sequence in the demethylation of DMA-*N*-oxide suggests that the flavoprotein is also involved in the *N*–*C*-transoxygenation of *N*-oxygenated aromatic amines. In this reaction, however, only an electron accepted from the *N*-oxide has to be transferred to P450 by the flavoprotein; as oxygen in reduced state is already present in the *N*-oxide molecule no extraneous electron donor is needed. The function of P450 in this reaction would be to accept the oxygen from the *N*-oxide, allowing an electron rearrangement towards the lone pair of the nitrogen followed by a loss of a labilized carbon-bound hydrogen; thereby an unpaired electron will appear on a carbon allowing a *C*-oxygenation[72,73]. The lack of response of *N*-oxide demethylation to high concentrations of MeHgOH indicated that such an electron transfer would be very insusceptible to electrophilic reagents (Fig. 6).

It cannot be decided whether one flavoprotein with two acceptor sites or two different flavoproteins are involved in the two proposed electron-transport functions, *i.e.* from amine and from NADPH respectively to P450 or to oxygen. The decreased DMA-*N*-oxide demethylation activity in the presence of NADPH shows, however, that the redox state at one of these functional sites affects the activity of the other. This favours the first alternative.

ZIEGLER AND PETTIT[23] observed that removal of 30 % of the flavin from pork-liver microsomes by acid-salt treatment completely abolished the *C*-oxygenation activity, while the *N*-oxygenation was still very active. This suggests that the enzyme may have two prosthetic groups; the flavin responsible for the NADPH–P450 electron transfer being more loosely attached than the flavin involved in the electron transfer to the oxygen-acceptor site of the flavoprotein. This does not contradict the possibility of one bifunctional flavoprotein, as such enzymes with two prosthetic groups are known[70]. SATO *et al.*[60] have shown that the steapsin-solubilized naphthoquinone-stimulated NADPH-oxidase, which they claim to be active in *C*-oxygenation of aromatic amines, contains two molecules of FAD per enzyme molecule. Experiments on pig-liver microsomes by ZIEGLER AND PETTIT[23] demonstrate that NADH is as active as NADPH in the *N*-oxygenation of DMA. Thus, the more strongly bound flavin responsible for electron transport to the oxygen acceptor site of the flavoprotein molecule itself would be less specific with regard to electron donor than the flavin which transfers electrons from pyridine nucleotide to P450.

In conclusion the evidence presented above and previously[72,73] infers that the flavoprotein enzyme included in the detoxication chain of liver microsomes mediates two electron flows to P450, one from the amine substrate (flavin I) and one from NADPH (flavin II). The pathway involving flavin I is insensitive to electrophilic agents (Fig. 6) and is interrupted only by disintegration of the detoxication chain (Fig. 5). The electron transport from pyridine nucleotide to P450 mediated by flavin II is stimulated by phosphate (*cf.* ref. 60) and inhibited by low concentrations of electrophilic agents, whereby *C*-oxygenation is also affected. In addition flavin I can accept electrons from pyridine nucleotides (with lesser specificity towards the electron donor than flavin II) and transfer them to the oxygen–amine complex thereby me-

diating *N*-oxygenation and the release of carbon radicals associated with this type of reaction. Possibly because of a more favorable competition for electron donor with flavin II this reaction is favored by low concentrations of electrophilic agents. Electron transfer to both flavins is inhibited by higher concentrations of electrophilic agents.

It should be emphasized that with DMA the inhibition of *C*-oxygenation by carbon radicals, formed in the *N*-oxygenation reaction, is an effect obtained under conditions which must be considered to be less physiological (*e.g.* fragmented and isolated endoplasmic reticulum and high O_2 pressure[33,78]) than those prevalent in living animals. An accumulation of DMA-*N*-oxide paralleling that obtained in the present system cannot be demonstrated when this monocyclic noncarcinogenic amine is administered *in vivo*[27,28]. However, *N*-oxygenated metabolites of polycyclic carcinogenic amines accumulate *in vivo* under conditions which eventually lead to tumour formation[38]. The close correlation of increased *N*-oxygenation/*C*-oxygenation ratio *in vitro* with conditions favoring tumour induction *in vivo*[11] and the formation of electrophilic metabolites from the carcinogenic aromatic amine 2-aminofluorene *in vitro*[51], indicates that the mechanisms for oxygenation of aromatic amines proposed here have relevance for the production of reactive intermediates initiating the tumour-inducing process in chemical carcinogenesis.

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EFFECTS *IN VITRO* OF 2-AMINOFLUORENE OR ELECTROPHILIC AGENTS ON HEPATIC MICROSOMAL *N*- AND *C*-OXYGENATION OF AROMATIC AMINES*

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SUMMARY

The carcinogenic potency of tumour-inducing aromatic amines is increased under the influence of various genetic, environmental and hormonal factors. This effect is associated with a shift in the detoxication pattern of aromatic amines *in vivo* as well as in hepatic microsomal systems from *C*- to *N*-oxygenation. It has been suggested that the general noxious character of the *N*-oxygenation pathway is due to a release of electrophilic metabolites associated with this process.

The electron transport from reduced nicotinamide adenine dinucleotide phosphate to P450, essential for *C*-oxygenation but not for *N*-oxygenation, is in rat-liver microsomes inhibited by electrophilic agents. It is shown in this report that in microsomes from guinea pigs, which are refractory towards the carcinogenic action of aromatic amines, this effect is not preferential for *C*-oxygenation; *N*-oxygenation is equally inhibited.

The carcinogenic amine 2-aminofluorene when added *in vitro* is several hundred times more effective in inhibiting microsomal *C*-oxygenation of aromatic amines than the previously studied noncarcinogenic amine dimethylaniline.

Adrenal-dependent stress, which can be induced by carcinogenic amines *in vivo*, amplifies the shift towards the deleterious *N*-oxygenation pathway caused by electrophilic agents in microsomal systems.

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Abbreviations: AAF, 2-acetylaminofluorene; acetoxy-AAF, *N*-acetoxy-2-acetylaminofluorene; AF, 2-aminofluorene; DMA, dimethylaniline; DMA-*N*-oxide, dimethylaniline-*N*-oxide; G-6-P, glucose 6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; *N*-hydroxy-AAF, *N*-hydroxy-2-acetylaminofluorene; *N*-hydroxy-AF, *N*-hydroxy-2-aminofluorene; MeHgOH, methylmercurihydroxide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

INTRODUCTION

The tumour-inducing capacity of hepatocarcinogenic aromatic amines is influenced by a variety of genetic, environmental and endocrine conditions[1–5]. The increased carcinogenic potency under the influence of these factors is associated with a shift in the metabolic pattern of aromatic amines *in vivo*[6–9] and *in vitro*[10] from *C*- to *N*-oxygenation.

In previous reports some evidence was presented suggesting that electrophilic carbon radicals produced during *N*-oxygenation are responsible for the general noxious character of this metabolic pathway[11,12]. When the noncarcinogenic, monocyclic amine DMA was metabolized by rat-liver microsomes at high substrate levels the *C*-oxygenation of this substrate was inhibited. The experiments indicated that this effect was due to the formation of electrophilic intermediates which blocked the electron transfer from NADPH to P450. A similar effect was obtained by addition of electrophilic agents to the incubation mixture. The preferential damage to the *C*-oxygenation function of the detoxication chain under these conditions resulted in a several times increased *N*-oxygenation/*C*-oxygenation ratio. It was of interest to relate these effects on rat-liver microsomes with effects on microsomes from guinea pig, a species which is refractory towards the carcinogenic action of aromatic amines[2,4].

The monocyclic amine DMA, used as a model in this and previous reports, does not exhibit carcinogenic properties, nor can any *N*-oxygenation of this amine be demonstrated *in vivo*[13,14]. Only under the less physiological conditions prevalent *in vitro*, do *N*-oxygenation and the production of noxious metabolites become apparent[11,12,15–17]. In contrast carcinogenic amines are *N*-oxygenated and form electrophilic metabolites even *in vivo*, as indicated by the urinary metabolite pattern and by the binding of these amines to nucleophilic centers in the cell[18]. It will be shown in the present report that the carcinogenic amine AF is several hundred times more effective than DMA in inhibiting *C*-oxygenation of DMA in microsomal systems. In guinea-pig microsomes the inhibitory effect of high concentrations of the DMA substrate as well as of minor amounts of AF is not preferential for *C*-oxygenation; *N*-oxygenation is equally affected and thus no shift towards this noxious metabolic pathway occurs.

Short-term treatment with carcinogenic amines *in vivo* involves an adrenal-dependent stress reaction in the liver cells affecting the protein-synthesizing and detoxicating functions of the endoplasmic reticulum[10,19–23]. This effect may be relevant for the carcinogenic action, as intact adrenals are a prerequisite for tumour induction with aromatic amines[1,5]. The present investigation includes some results indicating that stress-induced adrenal stimulation *in vivo* amplifies the shift towards the pernicious *N*-oxygenation pathway caused by electrophilic agents *in vitro*.

Preliminary presentations of some of these data have been published[19,20].

MATERIALS AND METHODS

Animals

Male rats (150–200 g) of a Sprague Dawley strain from Anticimex AB, Rotebro (Sweden), and male guinea pigs (300–350 g) of a local breed were used. The rats were fed Anticimex rat pellets No. 210; the guinea pigs were maintained on Fors rabbit pellets (Fors AB, Stockholm, Sweden), fresh swedes and hay. The animals were starved 16–20 h before being killed.

Chemicals

AF was synthesized as described previously[21]; acetoxy-AAF was synthesized according to LOTLIKAR *et al.*[24]. G-6-P, G-6-PDH and NADP were obtained from Sigma Chemical Co., St. Louis, Mo.; MeHgOH from Casco AB, Stockholm (Sweden); Celite (diatomaceous earth) from John's Manville International Corp., New York, N.Y.; Ficoll from Pharmacia AB, Uppsala (Sweden).

Pretreatment of animals

The Celite powder was suspended in 1 ml physiological saline and injected intraperitoneally.

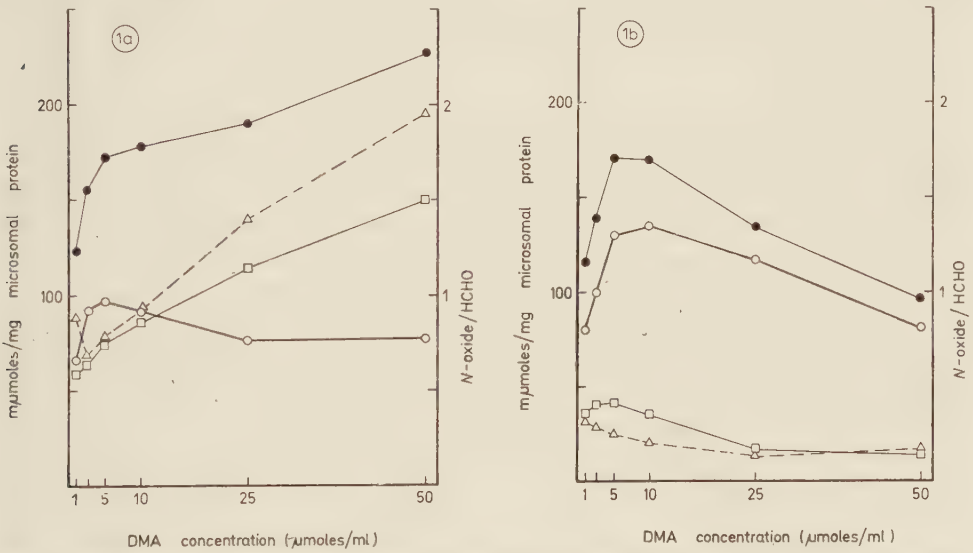

Fig. 1. Comparison of oxygenation pattern of DMA by (*a*) rat- and (*b*) guinea-pig-liver microsomes. Effect of substrate concentrations on *N*-demethylation (mμmoles HCHO/mg microsomal protein; ○), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein; □), total oxygenation (mμmoles HCHO + DMA-*N*-oxide/mg microsomal protein; ●) and *N*-oxygenation/*N*-demethylation ratio (△) of DMA. Washed microsomes were prepared in 0.2 *M* potassium phosphate buffer pH 7.5. All incubations were for 40 min at 35° and contained in a final volume of 2 ml: 0.1 μmoles NADP, 10 μmoles G-6-P, 0.25 Kornberg Units G-6-PDH, 120 μmoles nicotinamide, 2.3 mg (rat) or 2.2 mg (guinea pig) microsomal protein, DMA as indicated and 200 μmoles/ml potassium phosphate buffer pH 7.5.

In vitro systems

Microsomes from rat or guinea-pig livers were prepared as described previously[10]. The homogenization medium was 0.2 *M* potassium phosphate buffer pH 7.5, unless otherwise mentioned.

The preincubation experiments followed a previously described technique[12] using citrate medium (0.1 *M* sodium citrate in 0.1 *M* potassium phosphate buffer pH 7.5) in both incubations.

The incubation system for oxidative *N*-demethylation and *N*-oxide formation contained, when not otherwise stated, in a final volume of 2 ml: 0.2 ml microsomal suspension (1.5–3 mg microsomal protein), 0.1 μmoles NADP, 10 μmoles G-6-P, 120 μmoles nicotinamide, 0.25 Kornberg Units G-6-PDH, 10 μmoles DMA and 200 μmoles/ml of potassium phosphate buffer pH 7.5. The incubations were carried out aerobically in a water bath with agitation at 35°. The reaction was terminated by the addition of 0.9 *M* perchloric acid.

Analytical methods

All analytical methods were as described in a previous report[10].

RESULTS

Effects of substrate concentration on DMA oxygenation by rat- or guinea-pig-liver microsomes

It was shown previously[11,12] that *C*-oxygenation of DMA by rat-liver microsomes is progressively inhibited at substrate concentrations above 5–10 μmoles/ml, whereas *N*-oxygenation increases linearly. Fig. 1 shows the patterns of DMA oxygenation of increasing substrate concentrations, obtained with rat- or guinea-pig-liver microsomes. Guinea-pig preparations were similar to rat-liver microsomal systems in being progressively inhibited in their *C*-oxygenation capacity at high substrate concentrations, but differed in that this effect was not preferential for *C*-oxygenation in the guinea-pig preparations; indeed *N*-oxygenation was slightly more inhibited. Thus, in contrast to the situation in rat microsomes, total oxygenation was reduced. The *N*-oxygenation/*C*-oxygenation quotient, which at optimal substrate levels is already markedly smaller in guinea-pig than in rat preparations[10], was as a result further decreased.

Effects of AF treatment in vitro on DMA oxygenation by rat- or guinea-pig-liver microsomes

When AF was added to rat-liver microsomal systems (Fig. 2) it inhibited *C*-oxygenation of DMA already at concentrations a hundred times lower than the lowest DMA concentration shown to inhibit this reaction (Fig. 1*a*, *cf.* ref. 5). The *N*-oxygenation/*C*-oxygenation quotient was with this AF concentration twice as high as in controls. With increasing AF concentrations the inhibition of *C*-oxygenation became more marked. The *N*-oxygenation showed a slight optimum at 0.05 μmoles AF/ml, decreasing very slightly at higher concentrations. The *N*-oxygenation/

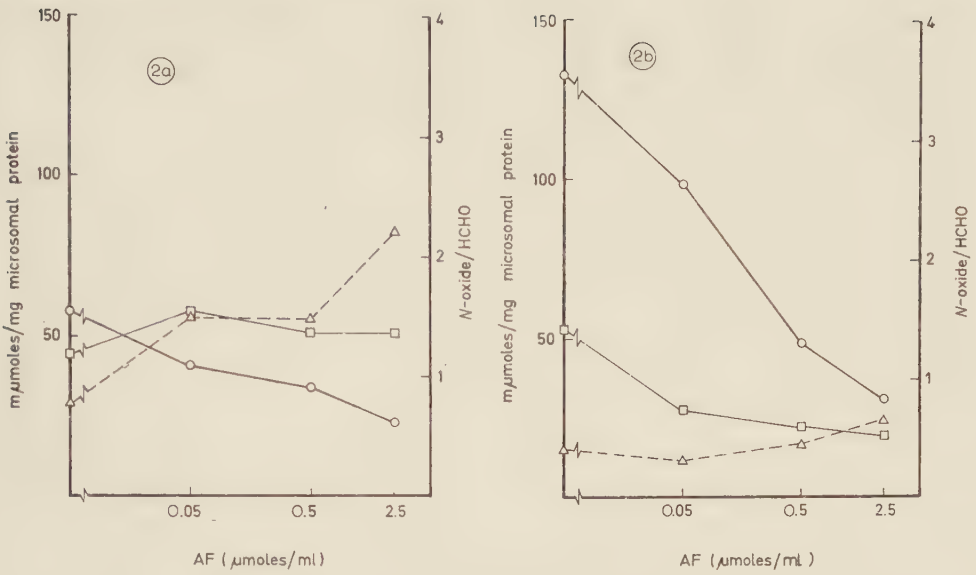

Fig. 2. Effect of AF on *N*-demethylation (mμmoles HCHO/mg microsomal protein; ○), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein; □) and *N*-oxygenation/*N*-demethylation ratio (△) of DMA by liver microsomes from (*a*) rats or (*b*) guinea pigs. Incubation conditions as in Fig. 1 with 10 μmoles DMA per 2 ml as substrate. Microsomal concentrations: 3.0 mg (rat) or 2.0 mg (guinea pig) protein/2 ml. AF as indicated.

C-oxygenation quotient at 0.05 μmoles AF was equal to that obtained with a 500 times higher DMA concentration. With increasing AF concentration this quotient increased but the difference from the effect of DMA was diminished; the 3-fold increase over normal obtained with 2.5 μmoles AF/ml could be obtained with just 20 times higher DMA concentration (*cf.* Fig. 1). The guinea-pig-liver microsomes exhibited the same differences from rat-liver microsomes in response to AF (Fig. 2*a* and *b*) as in the experiments with increasing DMA concentration (Fig. 1); *N*- and *C*-oxygenation showed a parallel inhibition. The *N*-oxygenation/*C*-oxygenation quotient thus remained low. At the highest AF concentration it increased somewhat; it did, however, not exceed the control value for rat-liver microsomes.

Effect of preincubation of rat- or guinea-pig-liver microsomes with AF

It was shown previously that the inhibitory effect of high DMA concentrations on *C*-oxygenation was not reversed by washing the rat microsomes[11,12]. Fig. 3 illustrates a similar experiment with AF. As mentioned previously the washing procedure *per se* increased *N*-oxygenation/*C*-oxygenation quotient somewhat[12]. The stabilizing effect of substrate (open symbols) on the microsomal enzymes during preincubation appearing as a decreased *N*-oxygenation and *N*-oxygenation/*C*-oxygenation quotient is also in agreement with previous results[12]. The addition of AF to the preincubation caused a drop in *C*-oxygenation in a second incubation with DMA as substrate. *N*-Oxygenation was just slightly affected. As a result the *N*-oxygenation/*C*-oxygenation quotient was strongly increased. The difference of guinea-

pig-liver microsomes from rat-liver microsomes also here paralleled those obtained with high concentrations of DMA (Fig. 1) or small amounts of AF in a single incubation (Fig. 2). *C*-Oxygenation and *N*-oxygenation were inhibited in parallel; the *N*-oxygenation/*C*-oxygenation quotient was virtually unchanged.

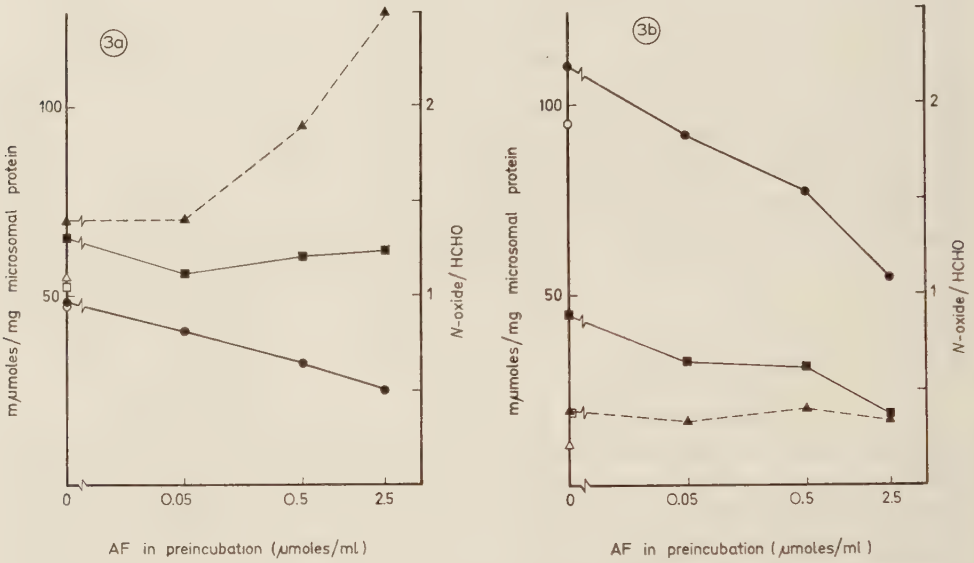

Fig. 3. Effect of preincubation of (*a*) rat-, or (*b*) guinea-pig-liver microsomes with various amounts of AF (filled symbols) or 10 μmoles DMA/2 ml (open symbols) on their activity in *N*-demethylation (mμmoles HCHO/mg microsomal protein; ●, ○), *N*-oxygenation (mμmoles HCHO/mg microsomal protein ■, □) and *N*-oxygenation/*N*-demethylation ratio (▲, △) of DMA during a second incubation after washing. Microsomes were prepared in 0.2 *M* phosphate buffer pH 7.5. Preincubations were for 30 min at 35° and contained in a final volume of 8 ml: 0.4 μmoles NADP; 40 μmoles G-6-P; 1.0 Kornberg Units G-6-PDH; 480 μmoles nicotinamide, 13.2 mg (rat) or 10.4 mg (guinea pig) microsomal protein, AF as indicated, 100 μmoles/ml sodium citrate pH 7.5 and 100 μmoles potassium phosphate buffer pH 7.5. Final incubations (incubation volume 2 ml) were under the same conditions as preincubation, with 10 μmoles/2 ml of DMA added as substrate and 1.6–1.8 mg (rat) or 1.5–1.6 mg (guinea pig) microsomal protein obtained from the preincubation mixture by centrifugation through 5 % Ficoll in 0.2 *M* potassium phosphate buffer pH 7.5.

Manifestation of preincubation effects

Fig. 4 shows the capacity of rat-liver microsomes to oxygenate DMA after various preincubation periods. When non-inhibitory amounts of DMA were present in the preincubation, there was only a minor effect of the preincubation *per se* within the time interval 0–30 min. The higher *N*-oxygenation/*C*-oxygenation quotient obtained in preincubation experiments as compared with a single incubation seems to be due to the washing procedure as it was evident even without preincubation. The inhibitory effect of AF on *C*-oxygenation and the associated increase in *N*-oxygenation/*C*-oxygenation quotient was obvious already after 2 min of incubation but became more manifest during 10–20 min incubation.

Effects of acetoxy-AAF on DMA oxygenation by rat- or guinea-pig-liver microsomes

Acetoxy-AAF has been shown to bind to nucleophilic centers in model systems

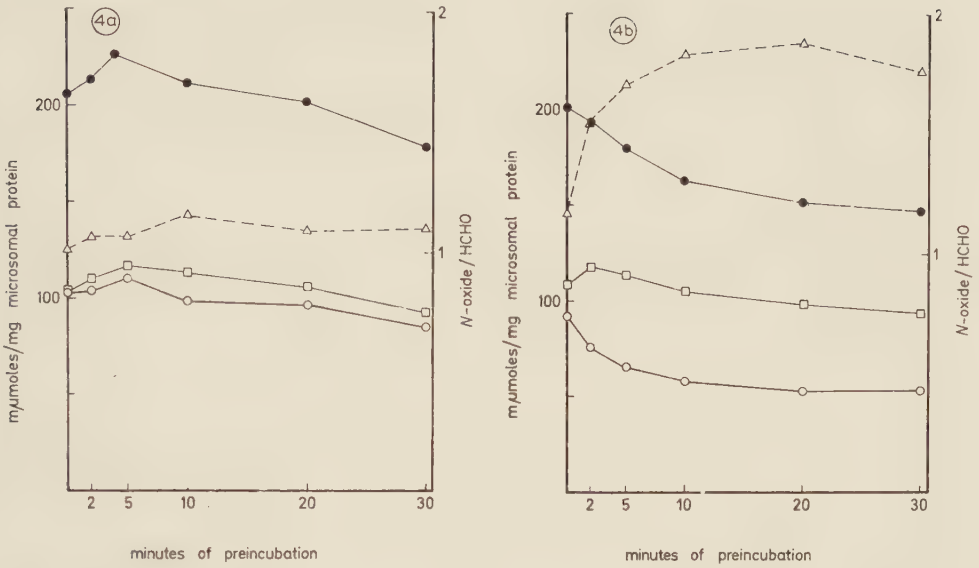

Fig. 4. Effect of preincubation of rat-liver microsomes for various periods with (*a*) 10 μmoles DMA/2 ml or (*b*) 1 μmole AF/2 ml on their activity in *N*-demethylation (mμmoles HCHO/mg microsomal protein; ○), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein; □), total oxygenation (mμmoles HCHO + DMA-*N*-oxide/mg microsomal protein; ●) and *N*-oxygenation/*N*-demethylation ratio; △) of DMA during a second incubation after washing. Preincubation as in Fig. 3 with 13.6 mg microsomal protein/8 ml and for time periods indicated. Final incubation as in Fig. 5 with 1.7–1.9 mg microsomal protein/2 ml.

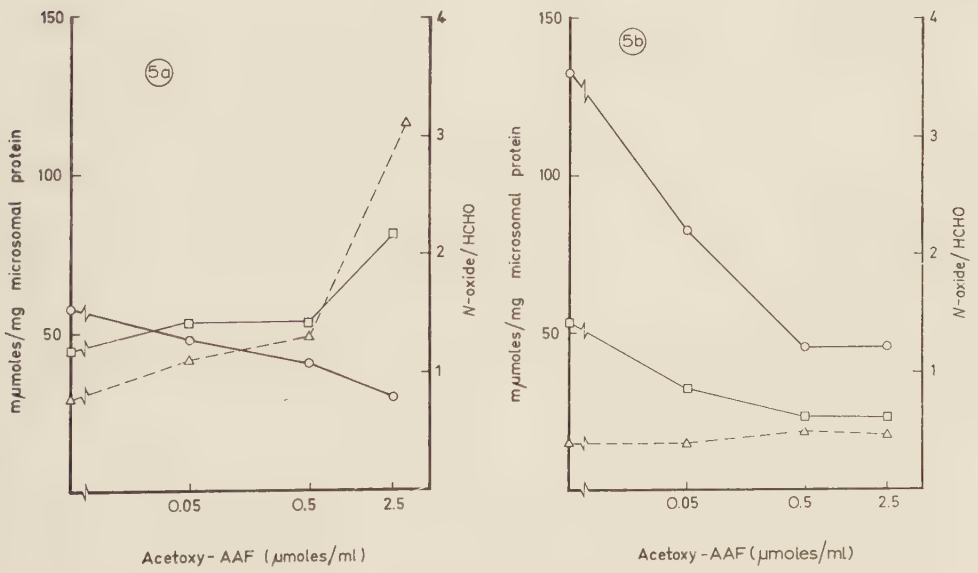

Fig. 5. Effect of acetoxy-AAF on *N*-demethylation (mμmoles HCHO/mg microsomal protein; ○), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein; □) and *N*-oxygenation/*N*-demethylation ratio (△) of DMA by liver microsomes from (*a*) rats or (*b*) guinea pigs. Incubation conditions as in Fig. 2. Microsomal concentrations: 3.0 mg (rat) or 2.0 mg (guinea pig) protein/2 ml; acetoxy-AAF as indicated.

independent of metabolic conversions[24,25]. The effect of this compound on DMA oxygenation by liver microsomes (Fig. 5) was very similar to that obtained with AF (Fig. 2). *C*-Oxygenation by rat-liver microsomes was progressively inhibited by increasing acetoxy-AAF concentrations. The *N*-oxygenation pattern was somewhat different from that obtained with AF; it was increased with higher acetoxy-AAF concentrations. As a result the *N*-oxygenation/*C*-oxygenation quotient with 2.5 μmoles/ml acetoxy-AAF was somewhat higher than with the corresponding AF concentration. Similar to AF, acetoxy-AAF affected *C*-oxygenation and *N*-oxygenation in parallel in guinea-pig-liver microsomes, thus leaving the low *N*-oxygenation/*C*-oxygenation quotient in these preparations virtually unchanged.

Synergistic effects of Celite in vivo treatment and MeHgOH in vitro on DMA oxygenation pattern

It was demonstrated previously that the effects of short-term treatment with carcinogenic amines include an adrenal-dependent stress response[21,22] giving rise to an increased DMA oxygenation associated with a shift in oxygenation pattern in favour of the *N*-oxygenation reaction[10,22]. This unspecific stress effect of carcinogenic amines is mimicked by an intraperitoneal injection of Celite[10,19–21]. In later reports a preferential inhibition of the *C*-oxygenation reaction with electrophilic agents added *in vitro* was demonstrated[11,12]. Fig. 6 demonstrates the synergistic effect of these factors. Fig. 6*a* illustrates the preferential inhibition of *C*-oxygenation by MeHgOH already demonstrated previously[11,12]. As is seen from Fig. 6*b* this

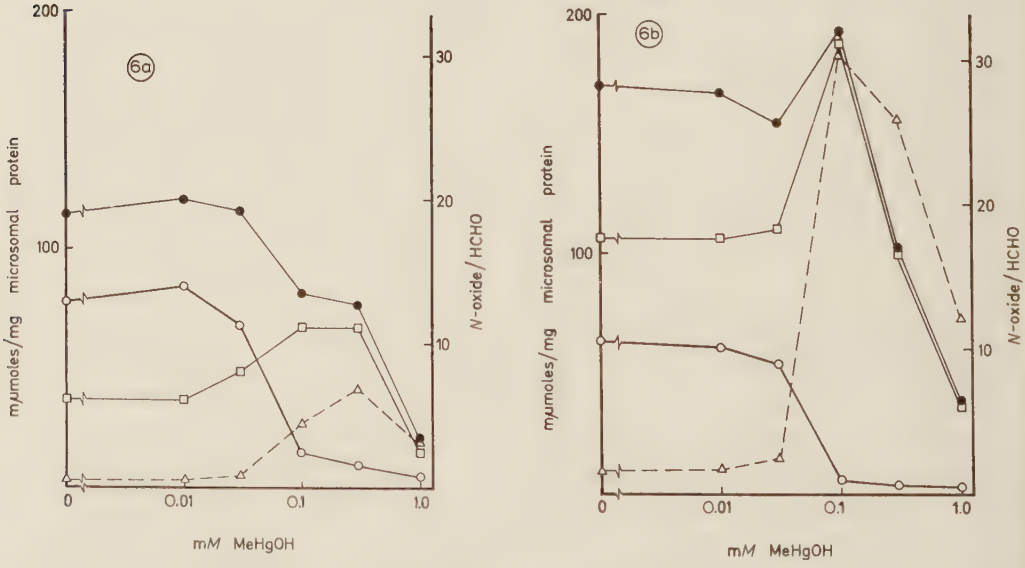

Fig. 6. Effect of MeHgOH on *N*-demethylation (mμmoles HCHO/mg microsomal protein; ○), *N*-oxygenation (mμmoles DMA-*N*-oxide/mg microsomal protein; □), total oxygenation (mμmoles HCHO + DMA-*N*-oxide/mg microsomal protein; ●) and *N*-oxygenation/*N*-demethylation ratio (△) of DMA by liver microsomes from (*a*) control or (*b*) Celite-treated (225 mg/kg body wt., 8 h) rats. Incubation conditions as in Fig. 2. Microsomal concentrations: 2.9 mg (control) or 3.5 mg (Celite) protein per 2 ml. MeHgOH as indicated.

effect was several times magnified by a previous *in vivo* treatment with Celite. At a MeHgOH concentration of 0.1 *M* when *N*-oxygenation reached optimum the *N*-oxygenation/*C*-oxygenation quotient was 60 times larger than in preparations from control animals incubated in absence of MeHgOH.

DISCUSSION

A fundamental event in the chemical carcinogenesis is the formation of anomalies in intracellular information transfer caused by the interaction of reactive metabolites of the carcinogen with macromolecules in the target cell[18,22,26–29]. In liver there is a close correlation between *N*-oxygenation of aromatic amines *in vivo*[6–9,30] and *in vitro*[10,22] on the one hand, and conditions giving rise to increased interference with ribosomal anabolic functions[21,22,31] and potentiation of the tumour-inducing properties of the carcinogenic aromatic amines on the other[1–5]. These facts support the view that microsomal *N*-oxygenation in contrast to *C*-oxygenation is a particularly pernicious metabolic pathway for aromatic amines, giving rise to reactive electrophilic metabolites, which combine covalently with cellular constituents[18]. Previous experiments[11,12] suggested that electrophilic carbon radicals produced during *N*-oxygenation of aromatic amines were responsible for the noxious character of this metabolic pathway. By interaction of these radicals with nucleophilic centers in the mechanism for electron transfer from NADPH to P450, the *N*-oxygenation and thereby also the level of carbon radicals is autocatalytically increased[12].

Guinea pigs are refractory towards the carcinogenic action of AF, AAF and aminoazo dyes[2,4] and *N*-oxygenate AF or AAF *in vivo* to a very limited extent[6,9]. Although guinea-pig-liver microsomes do *N*-oxygenate DMA to a certain extent, the *N*-oxygenation/*C*-oxygenation quotient is only one third of that of rat-liver microsomes, at a DMA concentration optimal for *C*-oxygenation[10]. This difference in oxygenation pattern between rat- and guinea-pig-liver microsomes is drastically amplified when the substrate concentration is increased (Fig. 1). The autocatalytically increased shift towards the pernicious *N*-oxygenation pathway, exhibited by rat microsomes under these conditions[11,12] does not occur in guinea-pig preparations. This is not due to a lack of response towards the inhibitory action of electrophilic metabolites on the electron transport from NADPH to P450; the *C*-oxygenation is inhibited as in rat microsomes. However, in contrast to this selective effect in rat microsomes, the microsomal *N*-oxygenation of guinea pig is inhibited in parallel with *C*-oxygenation when the DMA concentration is increased above levels optimal for this latter reaction.

Thus, the guinea pig because of greater susceptibility towards the deleterious action of the electrophilic carbon radicals avoids the preferential inhibition of NADPH–P450 electron transport which is a prerequisite for the autocatalytically magnified leakage of these noxious metabolites[11,12].

The adrenal glands play an important role in the early phase of hepatocarcinogenesis with aromatic amines[1,5]. Thus adrenalectomy, before or during the first week of carcinogen treatment abolishes the tumour-inducing properties of carcinogenic aromatic amines; on the other hand administration of corticosteroids simul-

taneously with the carcinogenic amines increases the tumour incidence and drastically shortens the latent period[1].

The response pattern of liver microsomes towards short-term *in vivo* treatment with AF or AAF involves a corticoid-dependent stimulation of protein-synthesizing activity[21,22] fairly soon overcompensating the inhibitory action of carcinogens on this function[31]. This effect, which can be mimicked by stressing the rats with an intraperitoneal Celite injection[21], is associated with a shift in oxygenation pattern towards *N*-oxygenation, caused by a preferential stimulation of this metabolic pathway[10,19,20]. It is demonstrated here that this preferential stimulation of *N*-oxygenation acts strongly synergistically with the inhibition of *C*-oxygenation by electrophilic agents (Fig. 6). The effect of prolonged AF treatment *in vivo* on the amine oxygenation pattern[10] could thus be interpreted as the result of two effects: (*1*) An inhibition by electrophilic carbon radicals of the vulnerable flavin which mediates NADPH–P450 electron transfer thus causing autocatalytically magnified leakage of these radicals, and (*2*) a stress response induced by the noxious metabolites involving a preferential stimulation of amine *N*-oxygenation, further reinforcing the deleterious effects of aromatic amines.

It should be noted here that the corticoid-dependent effect of aromatic amines does not necessarily act *via* the normal stress circuit, median-eminence–pituitary gland–adrenal cortex[32,33]. An increased level of corticosteroids in the liver cells could also be induced by a direct inhibitory action of the carbon radicals on microsomal enzymes converting corticosteroids to tetrahydro derivates. This step in the hepatic corticosteroid metabolism, which is very susceptible to electrophilic agents[34], is a prerequisite for the conjugation of corticosteroids to excretable conjugates[33].

In this and previous studies[10–12,19,20] on the influence of various *in vivo* and *in vitro* conditions on the microsomal oxygenation pattern of aromatic amines, the noncarcinogenic monocyclic amine DMA rather than a carcinogenic amine was selected as a substrate, thus obtaining controls with measurable oxygenation of the substrate (*cf.* ref. 35). The carcinogenic amine AF when added to a microsomal DMA oxygenation system caused a marked shift in the oxygenation pattern, even when added in minor amounts; to obtain a doubling of *N*-oxygenation/*C*-oxygenation quotient 500 times less AF than DMA was needed. As for DMA, the inhibitory effect was not reversed by washing the microsomes and thus was not due to an inhibitor present in the medium, but rather to irreversible damage to a component in the *C*-oxygenation mechanism by a reactive metabolite of the carcinogen. This metabolite seems to be formed continuously during incubation as, even if the inhibition is evident already after some few minutes of preincubation, it becomes more marked as the incubation proceeds.

Acetoxy-AAF exerts a similar influence on the microsomal oxygenation pattern. Acetoxy-AAF has been suggested as an ultimate carcinogenic metabolite formed by esterification of *N*-oxygenated derivatives in the cell[24]. This assumption is based on the findings that *N*-hydroxy-AF or *N*-hydroxy-AAF do not inhibit microsomal protein-synthesizing activity *in vitro*[36], and that acetoxy-AAF binds to guanine and methionine sulfur in model systems at physiological pH yielding the same

products as those obtained with AAF *in vivo*[24,25]. However, no esterification mechanism of this kind is known[24], and thus these experiments should be regarded as evidence that acetoxy-AAF spontaneously forms a reactive compound identical to the reactive metabolite obtained during the enzymatic metabolism of AF or AAF. In line with the idea of monovalent electron transfers proposed by MICHAELIS AND SCHUBERT[37], HORNER AND KIRMSE's[38] work indicates that acetoxy-AAF forms spontaneously an electrophilic carbon radical. Thus the binding of acetoxy-AAF in model systems to the same nucleophilic centers in guanine and methionine, as AF or AAF does in intact animals, supports the view that the reactive metabolites of carcinogenic amines are carbon radicals released during the *N*-oxygenation reaction. However, indirect evidence for the formation of other esters of *N*-hydroxy-AAF has been presented[39].

The experiments with AF and acetoxy-AAF showed the species specificity in oxygenation pattern already indicated in the experiments with the noncarcinogenic DMA; guinea-pig microsomes did not exhibit a shift towards *N*-oxygenation, when these substances were added to the incubation mixture (Figs. 2*b* and 5*b*), nor when the microsomes were preincubated with AF (Fig. 3*b*). As in the DMA experiments (Fig. 1) this is due to a higher sensitivity of the flavin enzyme towards the electrophilic intermediates and not to an insensitivity of P450 towards these metabolites.

The lack of reactivity of the *N*-oxygenated compounds themselves[24,25,36] has been taken as evidence by some authors that a further metabolic conversion is needed in order to obtain the ultimate carcinogenic metabolite. In addition to the esters discussed above, further oxidation to nitroso compounds has been suggested[35,36,40]. Nitrosofluorene in contrast to *N*-OH-AAF inhibits protein-synthesizing functions when added to cell-free systems[36]. However, the conversion of the *N*-hydroxylated metabolite to nitroso derivative is not shown to be an enzymatic process[35] and thus this metabolite in contrast to the carbon radicals is not necessarily formed in close proximity to the ribosomes *in vivo*; this strict localization of the site of formation of the reactive metabolites is indicated by the fact that binding of carcinogens *in vivo* primarily occurs in the material recovered in the microsomal and nuclear fractions[27,28,41].

The carcinogenicity of the *N*-oxygenated compounds has also been regarded as support for a further metabolic product being the ultimate carcinogen[40]. However, the work of IDE *et al.*[42] affords an alternative explanation for this effect; due to metabolic hydrolysis of the *NO*-glucuronide conjugates of hydroxylamines, this aglycone would remain longer in the body than the aglycones of *O*-glucosiduronic acid.

In conclusion the experiments presented above strongly reinforce the view[12] that the formation of electrophilic metabolites, suggested by HADDOW[43] and BOYLAND *et al.*[44] to be the actual carcinogens, is associated with the *N*-oxygenation reaction. Thus, the carcinogenic potency of aromatic amines can be attributed to their conversion to electrophilic radicals by enzymes in the endoplasmic reticulum of liver cells. Whether this property, inherent in their molecular configuration, finds expression is determined by genetic, hormonal and environmental factors affecting the conditions under which the metabolism proceeds.

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STUDIES ON LUNG TUMOURS

I. METHYLATION OF DEOXYRIBONUCLEIC ACID AND TUMOUR FORMATION FOLLOWING ADMINISTRATION OF DIMETHYLNITROSAMINE TO MICE

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SUMMARY

Methylation of lung and liver DNA was studied as a function of time in two inbred strains of mice with widely different susceptibilities to tumour formation by dimethylnitrosamine (DMNA). In GR males DMNA administration resulted in a high lung-tumour incidence and a very low number of liver tumours, whereas in $C3H_f$ males a high liver-tumour incidence and a very low number of lung tumours were found under the same conditions. No strain differences were demonstrable in the amounts of radioactivity in lung, liver and three other organs after one [^{14}C]DMNA injection. There were no strain differences in the amounts of 7-methylguanine present in lung or liver DNA from 5 h to 2 weeks after the injection of [^{14}C]DMNA. Methylated guanine was rapidly lost from DNA with time. Radioactivity of injected [^{14}C]DMNA was also associated with the guanine and adenine skeletons; in the DNA of the susceptible lung the specific radioactivities of both purines were appreciably higher than in the DNA of the resistant lung.

Pretreatment of mice with unlabelled DMNA in their drinking water gave rise to considerable changes in the methylation of lung, liver and kidney DNA by a subsequent dose of [^{14}C]DMNA. The results are discussed in relation to the possible significance of DNA methylation in the formation of tumours.

INTRODUCTION

Many nitrosamines are carcinogenic and possess high organ specificity (DRUCKREY *et al.*[1]). One of them, DMNA has been shown to be a very potent carcinogen for rat liver and kidney by MAGEE AND BARNES[2,3] and for rat lung by ZAK *et al.*[4]. *In*

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Abbreviations: DMNA, dimethylnitrosamine; 7-MeG, 7-methylguanine.

vivo, DMNA is enzymatically demethylated (MAGEE AND VANDEKAR[5], BROUWERS AND EMMELOT[6], HULTIN *et al.*[7]) and the monomethylated product yields, without diazomethane as an intermediate step (LIJINSKY *et al.*[8]), a methyl-carbonium ion which methylates cellular constituents by electrophilic attack. The major product of nucleic acid methylation by DMNA is 7-MeG (MAGEE AND FARBER[9]), small amounts of other methylated bases, *i.e.* 1-methyladenine, 3-methyladenine and 1-methylcytosine, having also been detected (LAWLEY *et al.*[10]).

In the present experiments the methylation of lung and liver DNA was studied under different carcinogenic conditions in two inbred strains of mice with widely different susceptibilities to tumour formation by DMNA. This study was aimed at obtaining more information about the role of the DNA in the mechanism of mouse lung and liver carcinogenesis by DMNA.

MATERIALS

Dimethyl ammonium chloride (laboratory reagent) was obtained from British Drug Houses (Poole, England). DMNA was supplied by Aldrich Chemical Co. (Milwaukee, Wis.), it was distilled before use and kept at −20° in the dark. [^{14}C]-Dimethyl ammonium chloride was obtained from Intern. Chemical and Nuclear Corp. (California) at specific activities of 2–49.6 mCi/mmole and a radiochemical purity of at least 99 %. After appropriate dilution with unlabelled dimethyl ammonium chloride, it was used for the preparation of [^{14}C]DMNA according to DUTTON AND HEATH[11]. The [^{14}C]DMNA solution was stored at 1–4° in the dark. The [^{14}C]-DMNA used in the first experiments was kindly supplied by Prof. P. N. MAGEE.

Animals

Male mice of the inbred strains C3H$_f$ and GR with different susceptibilities to the development of spontaneous lung tumours, as found by BENTVELZEN AND SZALAY[12], were used and received drinking water and standard laboratory diet (Hope Farms, Woerden, The Netherlands) *ad libitum*, unless otherwise stated. The animals were housed on softwood bedding (*cf.* VESELL[13]) and were subjected to a light–dark regimen and injected with DMNA between 2 and 4 h after the start of the 11-h light period (08.00 a.m.).

Tumour formation following DMNA administration

50 male GR and 50 male C3H$_f$ mice, two months old, were given DMNA in their drinking water. Because of toxicity the DMNA concentration was lowered after one month from 10 p.p.m. to 1 p.p.m. This lower concentration was continued for two months followed by a return to normal drinking water until killed. At age 26 weeks 30 mice of each strain were autopsied, followed by the remaining mice at one year.

In another series 3 groups of (12–17) male mice received, respectively, one DMNA injection (GR, 7 mg/kg body wt.; C3H$_f$, 10 mg/kg body wt.), 13 weekly DMNA injections (both strains 7 mg/kg body wt.) or (only GR males) 13 weekly

injections of physiological saline; injections were intraperitoneally. All mice were of the same age (15 weeks) at the first injection and were autopsied at age 38 weeks. Tumours on the lung surface were counted with the aid of a dissecting microscope (magnification × 10); only nodules with a diameter of 0.1 mm or larger were counted.

Distribution of radioactivity after one [^{14}C]DMNA injection

Groups of 4 mice, about 15 weeks old, received an intraperitoneal injection of 0.1 ml [^{14}C]DMNA solution in physiological saline (7 mg DMNA/kg body wt., 0.7–7 μCi/kg body wt.). The mice were killed by decapitation after 0.5, 1.5, 5 or 48 h. Lung, liver, kidney and spleen were collected and frozen on solid CO_2. Blood was collected in tubes with heparin, stored at 4° and within 1 h samples were withdrawn. To determine the radioactivity of the tissues, the method of HERBERG[14] was slightly modified by homogenizing tissues with hyamine hydroxide (15 mg tissue/ml; 1 *M* methanolic hydroxide of hyamine 10-X, Packard Instrument Cy., Ill.), and dissolving by gently shaking at 50° for at least 24 h. 3.5-ml samples of this solution were treated dropwise with 8 drops 30 % aqueous hydrogen peroxide and 15 ml scintillator solution (toluene–PPO–POPOP) was added. To determine acid precipitable radioactivity, tissues were homogenized with water (15 mg tissue/ml), trichloroacetic acid was added until a final concentration of 10 %, the precipitate was sedimented, washed with 10 % trichloroacetic acid and centrifuged again. The precipitates were dried under reduced pressure and dissolved in hyamine hydroxide as described before. Samples were counted in a liquid-scintillation spectrometer (TRI-CARB model 4312 or model 3380 with an automatic activity analyzer). Radioactivity was expressed as d.p.m./mg fresh wt. of tissue and corrected for the specific radioactivity of the injected [^{14}C]DMNA.

Methylation experiments

Groups of 20 or 60 mice, 13–17 weeks old, received one intraperitoneal injection of 0.1 ml [^{14}C]DMNA solution in saline (7 mg DMNA/kg body wt., 30–150 μCi/kg body wt.). The mice were killed by decapitation after 5 h, 48 h, 2 or 8 weeks. Lung, liver and in some cases kidney were collected, frozen on solid CO_2, stored at −20° or used directly. The first two groups of mice were fasted from 19 h before injection until death. The remaining groups, assayed 2 or 8 weeks after injection, were allowed food from 48 h after injection until 19–24 h before killing. In another series, 4 groups of 60 mice each received 10 p.p.m. unlabelled DMNA in their drinking water during 6 weeks. These mice were killed 48 h after one [^{14}C]DMNA injection.

DNA was originally isolated according to KIRBY[15] or in most experiments, by the more recent method for the preparation of 22–24S DNA according to KIRBY AND COOK[16]. The method of KIRBY was slightly modified as described earlier (SLUYSER AND DEN ENGELSE[17]). Mean yields of DNA as isolated by the method of KIRBY AND COOK[16] from lung and liver tissue were about 130 mg/100 g tissue, the older method of KIRBY[15] giving slightly lower results. This amounts to about 50 % of the DNA content in the case of liver[18] and 20 % of the DNA content in the lung (unpublished results). DNA was assayed according to SEIBERT[19], RNA according to

CERIOTTI[20] and protein by the method of LOWRY[21]. For measurement of the radioactivities of the DNA bases, the DNA was precipitated quantitatively by calf-thymus lysine-rich histone prior to base separation in order to remove most of the contaminating RNA (see ref. 17).

Base separation; determination of radioactivity

The DNA–histone precipitate was dissolved and hydrolyzed with 96 % formic acid at 175° for 35 min. The solvent was evaporated and the residue dissolved in 0.5 *N* HCl. Bases were separated on a Dowex-50W X8 column (15 × 1 cm) with the aid of 0.5 *N*, 1 *N* and 2 *N* HCl. Unlabelled 7-MeG, prepared according to JONES AND ROBINS[22] was added in order to identify the radioactive fraction from the DOWEX column eluted between guanine and adenine. The peaks of radioactivity and optical density coincided exactly. Small amounts of radioactive substances were also eluted with the guanine and adenine fractions. These radioactivities were quantitatively measurable only in the case of lung DNA because of the high doses of [^{14}C]DMNA used in these experiments.

Aliquots were evaporated at reduced pressure in a waterbath of 55° maximal. Because of the possibility that part of the radioactivity of the 7-MeG fraction might be due to 3-methyladenine (KRIEK AND EMMELOT[23]) thin-layer chromatography on cellulose was performed with ethanol–28 % ammonia–water (80:2:18, v/v/v) by which the two methylated bases are separated.

For identification of the guanine fraction thin-layer chromatography on cellulose was performed with propan-2-ol–11.6 *N* HCl–water (680 ml:176 ml to 1000 ml final volume); *n*-butanol saturated with water–conc. ammonia (100:1, v/v) was used for the adenine fraction. The collected fractions were evaporated and the residue dissolved in 0.5 ml hyamine hydroxide. 15 ml scintillator solvent (toluene–PPO–POPOP) was added and samples were counted as indicated before. In some cases the method of HATTORI *et al.*[24] was used. Radioactivity was expressed as d.p.m. × mg DMNA injected/$E_{260\ m\mu}$ × μCi DMNA injected; in the case of 7-MeG the radioactivity was related to the extinction of guanine.

TABLE I

LUNG AND LIVER TUMOURS FOLLOWING DMNA IN DRINKING WATER

	Age: *6 months*		*12 months*	
Strain	GR	$C3H_f$	GR	$C3H_f$
Total number of animals	30	30	20	20
Number of lung-tumour bearing animals	30	6	20	18
Mean number of lung tumours per animal	154 ± 29	0.4	143 ± 48	1.1
Number of liver-tumour bearing animals	0	1	2	9
Mean number of liver tumours per animal	0.0	0.5	0.1	0.5

RESULTS

Tumour formation in mice receiving DMNA in their drinking water

As shown in Table I, GR males were strikingly more sensitive than $C3H_f$ males with regard to the formation of lung tumours following DMNA treatment. Liver-tumour formation showed the reverse incidence in the two strains.

TABLE II

LUNG-TUMOUR FORMATION IN GR MALES FOLLOWING INTRAPERITONEAL DMNA INJECTIONS

Injections	*Total number of animals*	*Number of tumour-bearing animals*	*Mean number of lung tumours per animal*
1 × DMNA	17	17	32
13 × DMNA	16	16	169
13 × Saline	13	3	0.2

Tumour formation following intraperitoneal DMNA injections

Because the induction of tumours by nitrosamines strongly depends on dose (RIOPELLE AND JASMIN[25]) and in all methylation experiments (below) one i.p. injection of [^{14}C]DMNA was given, it was of interest to see whether the tumours could also be induced by a single i.p. injection of DMNA. In GR males a single injection or 13 weekly injections of DMNA (7 mg/kg body wt.) resulted in high numbers of lung tumours (Table II). No liver tumours were detected macroscopically. In the once injected group one salivary gland adenoma was found. In the group which received 13 injections one salivary gland adenoma, one adrenal tumour and one unidentified abdominal tumour were found. In the control group no tumours were found besides 3 lung tumours. Of 30 $C3H_f$ males receiving one injection of 10 mg DMNA/kg body wt. 11 animals died within one week. In the surviving ones no tumours could be detected at autopsy. After 13 injections (7 mg DMNA/kg body wt.) 3 beginning lung papillomas were detected. From these figures it follows that the same relative lung-tumour incidences are found as in the experiments in which the mice received DMNA in their drinking water. The present results do not allow a conclusion about the liver-tumour formation by DMNA since hepatomas usually appear at a later stage than that at which these mice were killed.

TABLE III

SPONTANEOUS LUNG AND LIVER TUMOURS IN MALE MICE

Strain	*Lung-tumour incidence*[a]	*Liver-tumour incidence*[a]
GR	40 % at 12 months	—[b]
$C3H_f$	8 % at 24 months	25 % at 21 months

[a] Percentage of animals bearing one or more lung, respectively liver tumours.
[b] No meaningful figures can be given in this case; see text.

Spontaneous tumour incidences

The spontaneous lung-tumour incidence in GR males is very high (Table III) in contrast with the rather low incidence in $C3H_f$ males. Spontaneous liver tumours occur rather frequently in $C3H_f$ males. These figures are derived from groups of at least 40 animals from the breeding colony of our institute. No meaningful figures can be given for the incidence of spontaneous liver tumours in GR males because these animals were autopsied at the age of 14 months or less. The first spontaneous liver tumours in $C3H_f$ males are found at 17 months. Spontaneous salivary gland or adrenal adenomas have never been observed in GR mice. Most of the spontaneous lung tumours as well as those formed after the administration of DMNA proved to be of the common papillomatous type. The histology of the liver tumours following DMNA administration did not differ from that of the spontaneously arising ones which are usually described as hepatomas.

Tissue distribution of radioactivity from [^{14}C]DMNA

Fig. 1 shows that there were no significant differences between the distribution of ^{14}C label in the two strains, either in the total tissue fractions or in the acid-precipitable fractions following one injection of labelled DMNA. The maximal radioactivity of the total tissue fractions precedes in most cases the maximal labelling of the acid-precipitable macromolecular fractions. The total, acid-precipitable and (by subtraction) acid-soluble radioactivities per unit fresh weight of tissue were considerably higher in the liver than in the other organs.

Methylation of lung, liver and kidney DNA

DNA of lung and liver was contaminated by 4–15 % RNA depending on the period between DMNA injection and death. The RNA content decreased with time after the DMNA injection. This result was found with both DNA isolation procedures. The bulk of contaminating RNA was removed by histone as described under *methylation experiments*, yielding a final DNA preparation with 2 % RNA or less. DNA usually contained less than 3 % protein.

The methylation of guanine at N-7 in mouse-lung and -liver DNA as a function of time after [^{14}C]DMNA injection is presented in Table IV. In a typical experiment it was shown by thin-layer chromatography (see under *base separation*) that the 7-MeG contained less than 2 % 3-methyladenine. Results are expressed in units of methylation (d.p.m. 7-MeG $\times$ mg DMNA injected/$E_{260\ m\mu}$(G) $\times$ μCi DMNA injected). One unit of methylation corresponds to 75 guanine residues methylated at N-7 out of 10^6 guanine residues or 0.34 mole 7-MeG/mole DNA (on the base of an arbitrarily assumed mol.wt. 6.7×10^6 for DNA). Neither at 5 h nor at later times were there any significant differences in the 7-MeG content of the lung DNA's of the two strains. The same holds true for liver DNA. From the figures in Table IV and also from some experiments at intermediate times ($C3H_f$-liver DNA contained 25.9 units of methylation 1.5 h after [^{14}C]DMNA injection; $C3H_f$-lung DNA contained 1.28 units of methylation 24 h after [^{14}C]DMNA injection) it follows that the rate of disappearance of 7-MeG from DNA *in vivo* is strongly time-dependent with half-life

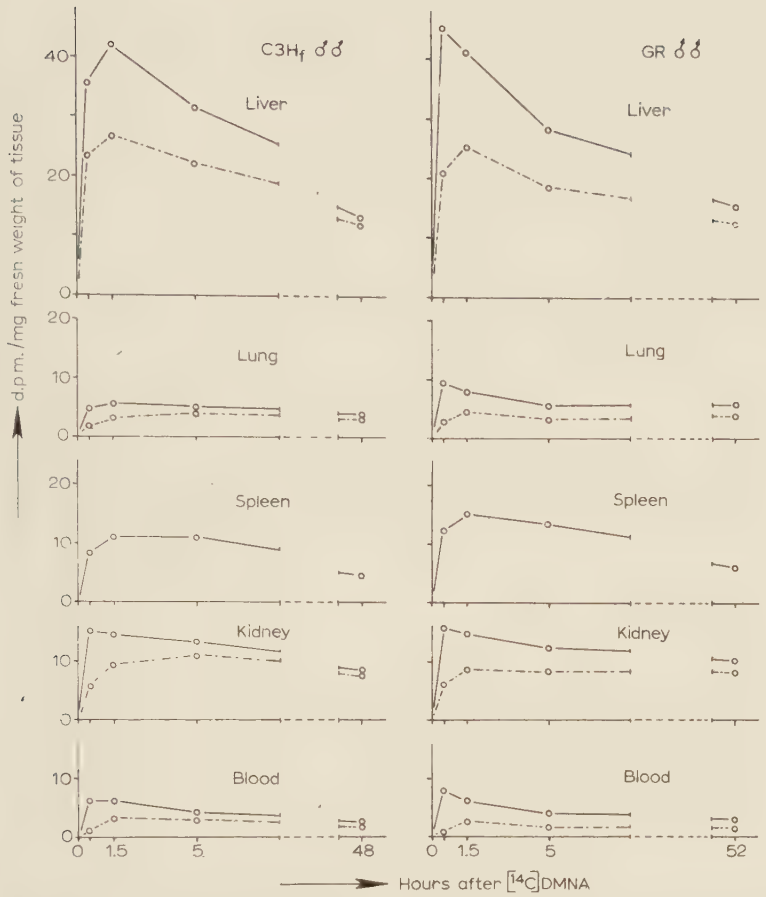

Fig. 1. Tissue distribution of radioactivity from [^{14}C]DMNA in the total tissue fractions (solid line) and in the acid-precipitable fractions (dashed line).

values for the 7-MeG content of DNA ranging from 10 h in the early period (1.5–5 h) after DMNA injection to 60–100 h in the late period (48 h–2 weeks). The data suggest that relatively more label is retained by lung than by liver DNA. In a preliminary experiment it was found that 8 weeks after [^{14}C]DMNA injection the 7-MeG content of lung and liver DNA of the $C3H_f$ males was about 0.01 unit of methylation corresponding to a loss of approx. 99 % of the 7-MeG found at 5 h after DMNA.

The methylation of lung, liver and kidney DNA following one i.p. injection of [^{14}C]DMNA into mice of both strains, pretreated with unlabelled DMNA in their drinking water as described before, is given in Table V together with the control 48-h values for untreated mice. In the pretreated mice the 7-MeG content of the liver DNA was decreased by a factor of about 2, whereas a rise of varying extent in the 7-MeG content of lung and kidney DNA was observed.

TABLE IV

METHYLATION OF GUANINE AT N-7 IN LUNG AND LIVER DNA AFTER ONE [^{14}C]DMNA INJECTION

Except for the lung data 2 weeks after DMNA, all figures are averages of 2 experiments with groups of 20–60 mice.

Tissue	*Time after [^{14}C]DMNA*	*Units of methylation*[a]	
		GR	*C3H$_f$*
Lung	5 h	1.43 ± 0.20	1.78 ± 0.25
	48 h	1.01 ± 0.06	0.91 ± 0.03
	2 wks	0.13	0.13
Liver	5 h	22.3 ± 3.8	20.7 ± 0.7
	48 h	10.5 ± 2.0	10.5 ± 1.7
	2 wks	0.67 ± 0.12	0.73 ± 0.04

[a] See text.

TABLE V

METHYLATION OF GUANINE AT N-7 IN LUNG, LIVER AND KIDNEY DNA 48 h AFTER ONE [^{14}C]DMNA INJECTION IN NORMAL MALES AND IN MALES PRETREATED WITH UNLABELLED DMNA IN THEIR DRINKING WATER

All figures are averages of 2 experiments with groups of 60 mice.

Strain	*Pretreated*	*Units of DNA methylation*[a] *in*		
		Lung	*Liver*	*Kidney*
GR	—	1.01 ± 0.06	10.5 ± 2.0	1.81 ± 0.10
	+	1.68 ± 0.07	4.28 ± 0.61	2.74 ± 0.28
C3H$_f$	—	0.91 ± 0.03	10.5 ± 1.7	1.57 ± 0.07
	+	1.76 ± 0.05	4.75 ± 0.14	4.29 ± 0.06

[a] See text.

Incorporation of radioactivity in purines of lung DNA

After column chromatography part of the injected [^{14}C] radioactivity was found in the guanine and adenine fractions of the isolated lung and liver DNA. Most probably this radioactivity is derived from [^{14}C]formaldehyde, produced by the enzymatic demethylation of [^{14}C]DMNA, being incorporated into newly synthesized purines (after conversion to formate), which are used for DNA synthesis. The data on lung adenine and guanine labelling are presented in Table VI. 48 h after injection, the purines of the GR lung contained twice the radioactivity associated with the corresponding purines of the C3H$_f$ lung. Preliminary experiments show that at later time periods (2 or 8 weeks) the specific activities were either somewhat increased or decreased as compared with the 48-h values. Thin-layer chromatography revealed that at least 66 % and 60 % of the radioactivity associated with the guanine and adenine fractions obtained from the Dowex column was present in these bases. 10–18 % of the radioactivity was associated with unidentified spots on both chromatograms, whereas the remainder of the radioactivity did not separate from the site of application.

TABLE VI

INCORPORATION OF [^{14}C]C_1-FRAGMENTS IN PURINES OF LUNG DNA AFTER ONE [^{14}C]DMNA INJECTION

Except for data 2 weeks after DMNA, all figures are averages of 2 experiments with groups of 60 mice.

Base	*Time after* [^{14}C]*DMNA*	*d.p.m.* (*G*) × *mg DMNA* / $E_{260\ m\mu}$ (*G*) × *μCi DMNA*	
		GR	*C3H*$_f$
Guanine	5 h	0.44 ± 0.04	0.25 ± 0.02
	48 h	1.00 ± 0.10	0.47 ± 0.05
	2 wks	0.72	0.65
	Time after [^{14}C]*DMNA*	*d.p.m.* (*A*) × *mg DMNA* / $E_{260\ m\mu}$ (*A*) × *μCi DMNA*	
		GR	*C3H*$_f$
Adenine	5 h	0.16 ± 0.03	0.10 ± 0.01
	48 h	0.54 ± 0.03	0.28 ± 0.04
	2 wks	0.77	0.31

DISCUSSION

In the present experiments the 7-methylguanine content of lung and liver DNA of male GR and C3H$_f$ mice, which differ in their susceptibility to tumour formation by DMNA, was measured following administration of [^{14}C]DMNA. The 7-MeG was rapidly lost from the DNA's and after 2 weeks only 9 % of the original content (at 5 h after injection) in the case of lung DNA and 3 % in that of liver DNA remained. Cellular damage (CRADDOCK[26]), spontaneous removal of 7-MeG (KRIEK AND EMMELOT[23]) or enzymatic repair (STRAUSS *et al.*[27], GOLDTHWAITE[28]) might be involved. As also observed by other investigators[29], the half-life of 7-MeG in DNA was strongly time-dependent. It is of interest that the half-life calculated for the late period of 48 h to 2 weeks after DMNA injection, is in good agreement with earlier results[23] obtained on the half-life (69 h) of the *in vitro* release of 7-MeG from diazomethane-treated salmon sperm DNA at 37° and pH 7.4.

Apart from N-7 methylation of guanine, radioactive carbon, presumably as formate resulting from the *N*-demethylation of DMNA, was also incorporated into the purine skeletons of lung DNA. A strain difference in the extent of labelling was apparent. Whether these findings reflect differences in lung purine and/or DNA synthesis, or in purine pools—whether as a consequence of DMNA or not—, is presently being studied.

Following pretreatment with unlabelled DMNA, a quantitative change in the methylation of liver (decreased), and lung and kidney (increased) DNA-guanine's by [^{14}C]DMNA as compared with the results without pretreatment was observed. These results differ from those obtained by CRADDOCK AND MAGEE[30] in the rat. The changes observed in our experiments might be due to a decreased *N*-demethyl-

ating activity in the mouse-liver endoplasmic reticulum membranes, resulting from DMNA action, and a consequently higher supply of DMNA to the other organs. Enzymatic experiments are currently being carried out to test this hypothesis.

In the present experiments no differences were apparent in the uptake of DMNA by, and the extent of, binding of the reactive DMNA derivative to the N-7 position of the DNA-guanine of the livers or lungs of GR and $C3H_f$ mice, up to 2 weeks after administration. Hence, neither the availability of DMNA, nor the metabolic conversion by *N*-demethylation (unpublished enzymatic experiments), the formation of 7-MeG in DNA or its removal from DNA, did differ for the respective tissues of the two mouse strains. Although it can not be excluded that for a given tissue of the two strains different gene segments have been affected, or that other alkylation reactions (*e.g.* at O-6, see LOVELESS[31]) are involved, the present results would suggest that alkylation of DNA-guanine at N-7 is not involved in carcinogenesis. In earlier experiments by LEE *et al.*[32] the organs showing relatively high levels of RNA-guanine methylation are those in which tumours are formed after DMNA administration. In recent experiments by SWANN AND MAGEE[33], and LIJINSKY AND ROSS[34], with nitrosamines and other alkylating agents, a correlation between alkylation of DNA and carcinogenesis was, however, less or not apparent.

It has generally been found that liver and skin carcinogens are covalently bound to the DNA of their target tissue (see BROOKES[35] for review). However, binding to non-target tissues or binding of selected non-carcinogens may also occur (*e.g.* GOSHMAN AND HEIDELBERGER[36], but compare VAN DUUREN[37] for dibenz-(a,c)-anthracene). If DNA binding of carcinogens is essentially involved in the mechanism of carcinogenesis, all the presently available results would imply that mere binding, as experimentally observed, is not sufficient to lead automatically to tumour formation. This view is in line with the concept that carcinogenesis is not a single, all-or-non event, but a multiphasic process. It is also for this reason that experiments which seek to correlate tumour formation in tissues other than the epidermis with binding of the carcinogen, following one non-carcinogenic dose, may be of limited significance. In the present experiments we have used doses of DMNA which can cause pulmonary tumours in one of the two mouse strains studied, whereas no differences in 7-MeG formation in the lung DNA of the two strains has been observed. However, it may be well that in the present case DMNA (or other carcinogens, like urethane, which shows a similar pattern of action, *cf.* BENTVELZEN AND SZALAY[12]) may not act as an inducer of the carcinogenic process as in the rat where the spontaneous tumour incidence is low or negligible[1], but rather as an accelerator of a genetically-linked endogenous process of tumour formation. Increase in number of tumours and decrease of their latent period by the carcinogen might be brought about either by an immunological effect or by a mitotic wave which allows the latent tumour cells to grow out. Activation of a latent virus might also be involved.

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ERRATUM

EUGENE J. BARRY, DANUTA MALEJKA-GIGANTI AND HELMUT R. GUTMANN, Interaction of aromatic amines with rat-liver proteins *in vivo*, III. On the mechanism of binding of the carcinogens, *N*-2-fluorenylacetamide and *N*-hydroxy-2-fluorenylacetamide, to the soluble proteins, *Chem.-Biol. Interactions*, 1 (1969/70) 139–155.

p. 139, summary, line 9 should read:
. . . formed *via* *N*-hydroxy-FAA. The methionine adducts were characterized by alkaline . . .

p. 141, paragraph 2, line 4: replace ref. 14 by ref. 11.

p. 141, line 5 from bottom should read:
. . . LOTLIKAR *et al.*[15]. 2-Nitro-[9-^{14}C]fluorene (0.086 g, $2.15 \cdot 10^9$ d.p.m./mmole) in a . . .

p. 143, legend to Fig. 1:
line 3: replace 1.4·108 by $1.4 \cdot 10^8$;
lines 9 and 10: replace μ by mμ.

p. 145, Table II, line 4, column 4 (In fraction 2):
replace 1560 ± 30 by 560 ± 30

p. 146, line 14 should read:
. . . of the aromatic amide, is converted to the acetate, phosphate or sulfate esters[6].

p. 149, Table V, footnote a, line 2 should read:
. . . at a concentration of 0.009 μmoles of compound/mg protein and with [9-^{14}C]FA at a concentration of 0.0045 μmoles of compound/mg protein . . .

p. 150, Table VI, footnote a, line 2 should read:
. . . of 0.009 μmoles/mg of protein . . .

p. 152, line 13 should read:
. . . of [9-^{14}C]FAA, 25–50% of the administered FAA was converted to *N*-hydroxy-FAA.

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Edited by

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